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(54) Title: ENZYME CATALYZED THERAPEUTIC AGENTS

(57) Abstract

This invention provides a method for identifying potential therapeutic agents by contacting a target cell with a candidate therapeutic agent which is a selective substrate for an endogenous, intracellular enzyme in the cell which is enhanced in its expression as a result of selection by biologic or chemotherapy. This invention also provides methods and examples of molecules for selectively killing a pathological cell by contacting the cell with a prodrug that is a selective substrate for an endogenous, intracellular enzyme. The prodrug is subsequently converted to a cellular toxin. Further provided by this invention is a method for treating a pathology characterized by pathological, hyperproliferative cells in a subject by administering to the subject a prodrug that is a selective substrate for an endogenous, overexpressed, intracellular enzyme, and converted by the enzyme to a cellular toxin in the hyperproliferative cell.

ENZYME CATALYZED THERAPEUTIC AGENTS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 U.S.C. § 119 (e) to U.S. Provisional Application Numbers 60/072,264; 60/076,950; and 60/108,634, filed January 23, 1998; March 05, 1998; and November 16, 1998, respectively. The contents of these applications are hereby incorporated by reference into the present disclosure.

TECHNICAL FIELD

The present invention relates to the field of drug discovery and specifically, the design of prodrugs which are substrates for an intracellular enzyme critical to resistance to therapeutics in pathological cells and converted to a cell toxin by the intracellular enzyme.

BACKGROUND

Throughout and within this disclosure, various publications are referenced by first author and date, patent number or publication number. The full bibliographic citation for each reference can be found within the specification or at the end of this application, immediately preceding the claims. The disclosures of these publications are hereby incorporated by reference into this disclosure to more fully describe the state of the art to which this invention pertains.

Cancer cells are characterized by uncontrolled growth, de-differentiation and genetic instability. The instability expresses itself as aberrant chromosome number, chromosome deletions, rearrangements, loss or duplication beyond the normal diploid

The classic alkylating agents are highly reactive compounds that have the ability to substitute alkyl groups for the hydrogen atoms of certain organic compounds. Alkylation of nucleic acids, primarily DNA, is the critical cytotoxic action for most of these compounds. The damage they cause interferes with DNA replication and RNA transcription. The classic alkylating agents include mechlorethamine, chlorambucil, melphalan, cyclophosphamide, ifosfamide, thiotepa and busulfan. A number of nonclassic alkylating agents also damage DNA and proteins, but through diverse and complex mechanisms, such as methylation or chloroethylation, that differ from the classic alkylators. The nonclassic alkylating agents include dacarbazine, carmustine, lomustine, cisplatin, carboplatin, procarbazine and altretamine.

Many clinically useful antitumor drugs are natural products of various strains of the soil fungus *Streptomyces*. They produce their tumoricidal effects by one or more mechanisms. All of the antibiotics are capable of binding DNA, usually by intercalation, with subsequent unwinding of the helix. This distortion impairs the ability of the DNA to serve as a template for DNA synthesis, RNA synthesis, or both. These drugs may also damage DNA by the formation of free radicals and the chelation of important metal ions. They may also act as inhibitors of topoisomerase II, an enzyme critical to cell division. Drugs of this class include doxorubicin (Adriamycin), daunorubicin, idarubicin, mitoxantrone, bleomycin, dactinomycin, mitomycin C, plicamycin and streptozocin.

Plants have provided some of the most useful antineoplastic agents. Three groups of agents from this class are the *Vinca* alkaloids (vincristine and vinblastine), the epipodophyllotoxins (etoposide and teniposide) and paclitaxel (Taxol). The *Vinca* alkaloids bind to microtubular proteins found in dividing cells and the nervous system. This binding alters the dynamics of tubulin addition and loss at the ends of mitotic spindles, resulting ultimately in mitotic arrest. Similar proteins make up an important part of nervous tissue; therefore, these agents are neurotoxic. The epipodophyllotoxins inhibit topoisomerase II and therefore have profound effects on cell function. Paclitaxel has complex effects on microtubules.

The antimetabolites are structural analogs of normal metabolites that are required for cell function and replication. They typically work by interacting with cellular enzymes. Among the many antimetabolites that have been developed and clinically tested are methotrexate, 5-fluorouracil (5-FU), floxuridine (FUDR), cytarabine, 6-mercaptopurine (6-

Table 1
Normal and Tumor Breast Epithelial Cells Are
Equally Sensitive to Doxorubicin Chemotherapy

Cell or Tissue	Number of Samples	Average IC_{50}
Normal Breast	13	14.8 ± 8.7ng/ml
Primary Carcinoma (UT)	19	11.4 ± 6.8 ng/ml
Metastatic Carcinoma (UT)	4	36 ± 26.3 ng/ml
Metastatic Carcinoma (Rx)	10	19.8 ± 12.7 ng/ml

From Smith et al. JNCI 74:341-347 (1985).

Hematologic toxicity is the most dangerous form of toxicity for many of the antineoplastic drugs used in clinical practice. Its most common form is neutropenia, with an attendant high risk of infection, although thrombocytopenia and bleeding may also occur and be life threatening. Chemotherapy may also induce qualitative defects in the function of both polymorphonuclear leukocytes and platelets. The hematopoietic growth factors have been developed to address these important side effects. Wilson, J.D. et al. (1991) and Dorr, R.T. and Von Hoff, D.D., eds. (1994).

Most of the commonly used antineoplastic agents are capable of suppressing both cellular and humoral immunity. Infections commonly lead to the death of patients with advanced cancer, and impaired immunity may contribute to such deaths. Chronic, delayed immunosuppression may also result from cancer chemotherapy.

The major forms of neurotoxicity are arachnoiditis; myelopathy or encephalomyelopathy; chronic encephalopathies and the somnolence syndrome; acute encephalopathies; peripheral neuropathies; and acute cerebellar syndromes or ataxia.

Many of the commonly employed antineoplastic agents are mutagenic as well as teratogenic. Some, including procarbazine and the alkylating agents, are clearly carcinogenic. This carcinogenic potential is primarily seen as delayed acute leukemia in patients treated with polyfunctional alkylating agents and inhibitors of topoisomerase II, such as etoposide and the anthracycline antibiotics. Chemotherapy has also been associated with cases of delayed non-Hodgkin's lymphoma and solid tumors. The present invention will minimize these effects since the prodrug will only be activated within tumor cells.

Table 2
Enzymes Overexpressed in Resistance to Cancer Chemotherapy

Enzyme	Biologic or Chemotherapy	Referenced (Examples)
Thymidylate synthase	Uracil-based Folate-based Quinazoline-based	Lönn, U. et al. *Cancer* 77:107,1996 Kobayashi, H. et al. *Jpn. J. Cancer Res.* 86:1014, 1995 Jackman, AL et al. *Anticancer Drug Des.* 10:573, 1995
Dihydrofolate reductase	Folate-based	Banerjee, D. et al. *Acta Biochem Pol.* 42:457, 1995 Bertino, J.R. et al. *Stem Cells* 14:5, 1996
Tyrosine kinases	TNF-alpha Multidrug resistance	Hudziak, R.M. et al. *PNAS* 85:5102, 1988 Stühlinger, M. et al. *J. Steroid Biochem* 49:39, 1994
MDR-associated proteins (ABC P-gp proteins)	Multidrug resistance	Simon, S.M. and Schindler, M. *PNAS* 91:3497, 1994 Gottesman, M.M. et al. *Annu. Rev. Genet.* 29:607, 1995
CAD*	PALLA**	Smith, K.A. et. al. *Philos. Trans. R. Soc. Lon. B. Biol. Sci.* 347:49, 1995 Dorr, R.T. and Von Hoff, D.D., eds. "Cancer Chemotherapy Handbook" 2nd ed. (Appleton and Lange 1994), pp. 768-773
Topoisomerase I (Colon & Prostate Cancers)	Camptothecin	Husain et al. *Cancer Res.* 54:539, 1994
Ribonucleotide reductase	Hydroxyurea	Wettergren, Y. et al. *Mol. Genet.* 20:267-85, 1994 Yen, Y. et al. *Cancer Res.* 54:3686-91, 1994

* CAD = carbamyl-P synthase, aspartate transcarbamylase, dihydroorotase
** PALA = N-(phosphonacetyl)-L-aspartate

Use of Prodrugs as a Solution to Enhance Selectivity of a Chemotherapeutic Agent

The poor selectivity of anticancer agents has been recognized for a long time and attempts to improve selectivity and allow greater doses to be administered have been numerous. One approach has been the development of prodrugs. Prodrugs are compounds that are toxicologically benign but which may be converted *in vivo* to therapeutically active products. In some cases, the activation occurs through the action of a non-endogenous enzyme delivered to the target cell by antibody ("ADEPT" or antibody-dependent enzyme prodrug therapy (U.S. Patent No. 4,975,278)) or gene targeting ("GDEPT" or gene

specifically converted to a cellular toxin by the intracellular target enzyme. In another aspect of this invention, the product of an initial reaction is subsequently fully activated by a common cellular enzyme such as an acylase, phosphatase or other "housekeeping" enzyme (Voet, et al. (1995)) or common cellular constituent (e.g., water) to release the toxic byproduct from the prodrug.

Further provided by this invention is a method for treating a pathology characterized by pathological, hyperproliferative cells in a subject by administering to the subject a prodrug that is a selective substrate for a target enzyme, and selectively converted by the enzyme to a cellular toxin in the hyperproliferative cell. The prodrugs of this invention may be used alone or in combination with other chemotherapeutics or alternative anti-cancer therapies such as radiation.

A further aspect of this invention is the preparation of a medicament for use in treating a pathology characterized by pathological, hyperproliferative cells in a subject by administering to the subject a prodrug that is a selective substrate for a target enzyme, and selectively converted by the enzyme to a cellular toxin in the hyperproliferative cell.

A still further aspect of this invention is a method for identifying the optimal therapeutic for a subject, by isolating cells overexpressing an endogeneous, intracellular enzyme and contacting the cells with at least one of the prodrugs of this invention, and then identifying which of the one or more prodrugs inhibits the proliferation or kills the cells, thereby indentifying the optimal therapeutic for the subject.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the development of resistance to anti-cancer modalities in cells, and the consequences.

Figure 2 schematically shows activation pathways of the prodrugs of this invention.

Figure 3 schematically shows the High Throughput Screen for prodrugs activated by intracellular enzymes important in drug resistance.

Figure 4 schematically shows how to find a lead human thymidylate synthase (TS) prodrug using TS-negative *E. coli* as the cell target.

Figure 5 shows an example of how to use this screen to simultaneously optimize the prodrug for reactivity to

R^4 can depart as an active metabolite of the anticancer agents Tepa or Thiotepa, a phosphoramide mustard, N-acetylsphingosine (C_2 ceramide, a tumor suppressor lipid) or hydroxyurea (an inhibitor of ribonucleotide reductase) upon release by TS. The leaving group R_1 can also be an α,α-dihalogenated ether, in which case it affords a carboxylic acid group when the α,α-dihalogenated alcohol released by TS undergoes hydrolysis. Thus, the leaving group R^4 can depart as a progenitor to fluoroacetate, fluorocitrate, malonic acid, methylmalonic avid, or 3-nitroproprionic acid, all potent inhibitors of oxidative phosphorylation.

Figure 7 shows TS Western blot of cell lines transfected by plasmid encoding neomycin resistance, with or without the HER-2 protooncogene. Lane (1) MCF7/HER2; (2) MCF7/neo; (3) MDA-MB-435/HER2; (4) MDA-NM-435/neo; (5) BT-20/HER2; (6) BT-20/neo.

Figure 8 shows reaction products of prodrug compounds with the enzyme thymidylate synthase.

DETAILED DESCRIPTION OF THE INVENTION

The invention is achieved by exploiting some of the key genomic and phenotypic changes intimately linked to resistance to biologic and chemotherapy of cancer cells. The invention provides a means for *in vivo* selectively inhibiting the growth and/or killing of cells which have undergone selection by exposure to cancer therapy (including biologic therapy such as tumor necrosis factor (TNF) or chemotherapy). (Refer to Table 2). As a result, certain enzymes which have been activated by mutation or gene amplification are resistant to initial or further therapy by the agent. Unlike prior art therapies directed to creating more potent inhibitors of endogenous, intracellular enzymes, this invention exploits the higher enzyme activity associated with therapy-resistant diseased cells and tissues versus normal cells and tissues and does not rely on inhibiting the enzyme. In one aspect, the tumor cells successfully treated by the prodrugs of this invention are characterized by enhanced target enzyme activity and therefore have a much higher potential to convert the prodrug to its toxic form than do normal cells which do not overexpress the target enzyme. The term "target enzyme" is used herein to define enzymes having one or more of the above noted characteristics.

drugs. While this application is specifically directed to cancer, a similar approach can be applied to enzymes encoded by human and animal pathogens, and in which the inhibitors have failed due to development of resistance.

Amplification of certain genes is involved in resistance to chemotherapy. Amplification of dihydrofolate reductase (DHFR) is related to resistance to methotrexate while amplification of the gene encoding thymidylate synthase is related to resistance to tumor treatment with 5-fluoropyrimidines. Amplification of genes associated with drug resistance can be detected and monitored by a modified polymerase chain reaction (PCR) as described in Kashini-Sabet, et al. (1988), U.S. Patent No. 5,085,983, or the method described herein. Acquired drug resistance can be monitored by the detection of cytogenetic abnormalities, such as homogeneous chromosome staining regions and double minute chromosomes both of which are associated with gene amplification. Alternative assays include direct or indirect enzyme activity assays and both of which are associated with gene amplification (e.g., Carreras & Santi (1995)); other methodologies (e.g. polymerase chain reaction, Houze, T.A. et al. (1997) or immunohistochemistry (Johnson, P.G. et al. (1997)).

Alternatively, the target cell is characterized as having inactivated tumor suppressor function, e.g. loss or inactivation of retinoblastoma (RB) or p53, known to enhance expression of TS (Li, W. et al. (1995)) or DHFR (Bertino, et al. (1996) and Li, W. et al. (1995)).

The prodrugs of this invention are useful to treat or ameliorate any disease wherein the disease-associated enzyme is associated with drug resistance to a chemotherapeutic whether due to loss of tumor suppressor functionality, *in vivo* selection by chemotherapy or a combination. This includes embodiments, where the enzyme is overexpressed, over-accumulated or activated in pathological cells versus normal cells, for example, the TS enzyme. Particularly excluded is the enzyme glutathione-S-transferase which has been shown to be occasionally elevated in some human tumors. Morgan, A.S. et al. (1998). The prodrugs of the subject invention are distinguishable on the basis that the target enzymes of this invention are commonly overexpressed, overaccumulated or activated in pathological cells versus normal cells. The most important principle which distinguishes the current invention from other approaches are:

method also identifies agents that inhibit the growth of cells or cell cycling of hyperproliferative cells, such as cancer cells. Other cells that are included are bacterial, yeast and parasitic cells which cause disease as a result of inappropriate proliferation in the patient. The agent is considered a potential therapeutic agent if cell proliferation, replication or cell cycling is reduced relative to the cells in a control sample. Most preferably, the cells are killed by the agent. The cells can be procaryotic (bacterial such as *E. coli*) or eucaryotic. The cells can be mammalian or non-mammalian cells, e.g., mouse cells, rat cells, human cells, fungi (e.g., yeast) or parasites (e.g., *Pneumocystis* or *Leishmania*) which cause disease.

As used herein, a "hyperproliferative cell" is intended to include cells that are de-differentiated, immortalized, neoplastic, malignant, metastatic or transformed. Examples of such cells include, but are not limited to a sarcoma cell, a leukemia cell, a carcinoma cell, or an adenocarcinoma cell. More specifically, the cell can be a breast cancer cell, a hepatoma cell, a dectectable cancer cell, pancreatic carcinoma cell, an oesophageal carcinoma cell, a bladder cancer cell, an ovarian cancer cell, a skin cancer cell, a liver carcinoma cell, or a gastric cancer cell. In an alternative embodiment, the target cell can be resistant to a drug or compound used to prevent or kill a cell infected with an infectious agent which is resistant to coventional antibiotics. Infectious agents include bacteria, yeast and parasites, such as trypanosomes.

Specific examples of target enzymes that are the subject matter of this invention are listed in Table 2 (above) or Table 3 (below). These enzymes are involved in resistance to chemotherapy, are endogeneously activated, overexpressed or over-accumulated in a cell characterized by resistance to cancer therapy and associated with a pathological or disease include, but are not limited to enzymes such as a member of the tyrosine kinase superfamily or an ATP-dependent MDR-associated protein, CAD, thymidylate synthase, dihydrofolate reductase, and ribonucleotide reductase. Table 3 provides a list of enzymes which may be targeted by this approach in infectious disease.

simultaneously compared to its effect on another enzyme or a corresponding enzyme from another species.

In another embodiment, a third target cell is used as a control because it receives an effective amount of a compound, such as, for example, the compounds shown below, which have been shown to be potent prodrugs. This embodiment is particularly useful to screen for new agents that are activated by thymidylate synthase.

In another embodiment, transformed cell lines, such as ras-transformed NIH 3T3 cells (ATCC, 10801 University Blvd., Manassas, VA 20110-2209, U.S.A.) are engineered to express variable and increasing quantities of the target enzyme of interest from cloned cDNA coding for the enzyme. Transfection is either transient or permanent using procedures well known in the art and described in Chen, L. et al. (1996), Hudziak, R.M. et al. (1988), or Carter, P. et al. (1992), and in the experimental section below. Suitable vectors for insertion of the cDNA are commercially available from Stratagene, La Jolla, CA and other vendors. The level of expression of enzyme in each transfected cell line can be monitored by immunoblot and enzyme assay in cell lysates, using monoclonal or polyclonal antibody previously raised against the enzyme for immuno-detection. See, e.g., as described by Chen, L. et al. (1996). The amount of expression can be regulated by the number of copies of the expression cassette introduced into the cell or by varying promoter usage. Enzymatic assays to detect the amount of expressed enzyme also can be performed as reviewed by Carreras, C.W. and Santi, D.V. (1995), or the method described in the experimental section below. Tumor cell lines can be selected to express enhanced levels of thymidylate synthase (*e.g.*, colon tumor cells, as described by Copur et al. (1995).

As noted above, cells containing the desired genetic deficiencies may be obtained from Cold Spring Harbor, the Agricultural Research Service Culture Collection, or the American Type Culture Collection. The appropriate strains can also be prepared by inserting into the cell a gene coding for the target enzyme using standard techniques as described in Miller (1992), Sambrook, et al. (1989), and Spector et al. (1998). Growth assays can be performed by standard methods as described by Miller (1992), Sugarman et al. (1985) and Spector et al. (1998).

It should be understood by those skilled in the art that the screen shown in Figure 3 can be applied broadly for the discovery of antibiotics. For example, thymidylate synthase from yeast could be substituted for that of *E. coli* in Figure 4. This would allow the

(published November 14, 1991). The leaving group of the candidate prodrug can be detectably labeled, e.g., with tritium. The target cell or the culture media is then assayed for the amount of label released from the candidate prodrug. Alternatively, cellular uptake may be enhanced by packaging the prodrug into liposomes using the method described in Lasic, D.D. (1996) or combined with cytofectins as described in Lewis, J.G. et al. (1996).

In a separate embodiment, cultured human tumor cells overexpressing the enzyme of interest i.e., target enzyme, are identified as described above. The cells are contacted with the potential therapeutic agent under conditions which favor the incorporation of the agent into the intracellular compartment of the cell. The cells are then assayed for inhibition of cellular proliferation or cell killing.

It should be understood, although not always explicitly stated, each embodiment can be further modified by providing a separate target cell to act as a control by receiving an effective amount of a compound, such as, for example, the compounds shown below, which have been shown to be potent prodrugs.

A high throughput screen to identify biologically active compounds is outlined in Figures 3, 4 and 5. The basis of the test is the ease of genetic manipulation and growth of *E. coli*, and similar single cell organisms (e.g. yeast), see Miller (1992) and Spector et al. (1998). The key step is removing the endogenous enzyme activity corresponding to an enzyme target for prodrug design. This can be done by any of the methods described by Miller (1992), Sambrook, et al. (1989) or Spector et al. (1998). These methods include chemical and biologic (e.g. viral or transponson insertional) mutagenesis, followed by an appropriate selection procedure. The TS negative (TS^-) cell then becomes a negative control for the identification of prodrugs that, when acted upon by thymidylate synthase, become cell toxins. A similar approach can be made with other cell types, e.g. other bacteria, yeast, or other selectable single cell organisms. In the assay, both control and recombinant organisms are compared for sensitivity to the test compounds. As will be understood by those skilled in the art, prodrugs which distinguish between species of enzyme can also be derived from this procedure. For example, otherwise identical cells expressing human and yeast enzymes can be used to detect antibiotic prodrugs which are preferentially toxic only to the cells expressing the yeast enzyme. In this way, novel and specific antibiotics can be discovered.

Another aspect of this invention is a method for treating a pathology characterized by hyperproliferative cells in a subject comprising administering to the subject a therapeutic amount of a prodrug that is converted to a toxin in a hyperproliferative cell by an endogenous intracellular enzyme as defined herein. In a preferred embodiment, the compound is selected from the compounds defined in the section "Prodrugs," Infra.

When the prodrug is administered to a subject such as a mouse, a rat or a human patient, the agent can be added to a pharmaceutically acceptable carrier and systemically or topically administered to the subject.

To determine patients that can be beneficially treated, a tumor sample is removed from the patient and the cells are assayed for the level of expression of the enzyme of interest. If the expression is above that expressed in normal cells so that a toxic amount of the prodrug would cause administered without undesirable side effects, then the tumor or cells are determined to be benefically treated and thus, the patient is suitable for the therapy of this invention. For example, if the target enzyme is expressed at least about 2 times and preferably about 3 times higher than normal cells, the patient is a suitable subject for the therapy method of this invention. Therapeutic amounts can be empirically determined and will vary with the pathology being treated, the subject being treated and the toxicity of the converted prodrug or cellular toxin.

When delivered to an animal, the method is useful to further confirm efficacy of the prodrug. As an example of an animal model, groups of nude mice (Balb/c NCR nu/nu female, Simonsen, Gilroy, CA) are each subcutaneously inoculated with about 10^5 to about 10^9 hyperproliferative, cancer or target cells as defined herein. When the tumor is established, the prodrug is administered, for example, by subcutaneous injection around the tumor. Tumor measurements to determine reduction of tumor size are made in two dimensions using venier calipers twice a week. Other animal models may also be employed as appropriate. Lovejoy et al. (1997) and Clarke, R. (1996).

Administration *in vivo* can be effected in one dose, continuously or intermittently throughout the course of treatment. Methods of determining the most effective means and dosage of administration are well known to those of skill in the art and will vary with the composition used for therapy, the purpose of the therapy, the target cell being treated, and the subject being treated. Single or multiple administrations can be carried out with the

Determining the optimal dosage will generally involve the balancing of the level of therapeutic benefit against any risk or deleterious side effects of the treatments of the present invention.

Ideally, the prodrug should be administered to achieve peak concentrations of the active compound at sites of disease. This may be achieved, for example, by the intravenous injection of the prodrug, optionally in saline, or orally administered, for example, as a tablet, capsule or syrup containing the active ingredient. Desirable blood levels of the prodrug may be maintained by a continuous infusion to provide a therapeutic amount of the active ingredient within disease tissue. The use of operative combinations is contemplated to provide therapeutic combinations requiring a lower total dosage of each component antiviral agent than may be required when each individual therapeutic compound or drug is used alone, thereby reducing adverse effects.

While it is possible for the prodrug ingredient to be administered alone, it is preferable to present it as a pharmaceutical formulation comprising at least one active ingredient, as defined above, together with one or more pharmaceutically acceptable carriers therefor and optionally other therapeutic agents. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient.

Formulations include those suitable for oral, recta, nasal, topical (including transdermal, buccal and sublingual), vaginal, parenteral (including subcutaneous, intramuscular, intravenous and intradermal) and pulmonary administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient with the carrier which constitutes one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then if necessary shaping the product.

Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets, each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or suspension in an aqueous or non-aqueous liquid; or as an oil-in-water liquid emulsion or a

If desired, the aqueous phase of the cream base may include, for example, at least about 30% w/w of a polyhydric alcohol, i.e., an alcohol having two or more hydroxyl groups such as propylene glycol, butane-1,3-diol, mannitol, sorbitol, glycerol and polyethylene glycol and mixtures thereof. The topical formulations may desirably include a compound which enhances absorption or penetration of the prodrug ingredient through the skin or other affected areas. Examples of such dermal penetration enhancers include dimethylsulfoxide and related analogues.

The oily phase of the emulsions of this invention may be constituted from known ingredients in an known manner. While this phase may comprise merely an emulsifier (otherwise known as an emulgent), it desirably comprises a mixture of at lease one emulsifier with a fat or an oil or with both a fat and an oil. Preferably, a hydrophilic emulsifier is included together with a lipophilic emulsifier which acts as a stabilizer. It is also preferred to include both an oil and a fat. Together, the emulsifier(s) with or without stabilizer(s) make up the so-called emulsifying wax, and the wax together with the oil and/or fat make up the so-called emulsifying ointment base which forms the oily dispersed phase of the cream formulations.

Emulgents and emulsion stabilizers suitable for use in the formulation of the present invention include Tween 60, Span 80, cetostearyl alcohol, myristyl alcohol, glyceryl monostearate and sodium lauryl sulphate.

The choice of suitable oils or fats for the formulation is based on achieving the desired cosmetic properties, since the solubility of the active compound in most oils likely to be used in pharmaceutical emulsion formulations is very low. Thus the cream should preferably be a non-greasy, non-staining and washable product with suitable consistency to avoid leakage from tubes or other containers. Straight or branched chain, mono- or dibasic alkyl esters such as di-isoadipate, isocetyl stearate, propylene glycol diester of coconut fatty acids, isopropyl myristate, decyl oleate, isopropyl palmitate, butyl stearate, 2-ethylhexyl palmitate or a blend of branched chain esters known as Crodamol CAP may be used, the last three being preferred esters. These may be used alone or in combination depending on the properties required. Alternatively, high melting point lipids such as white soft paraffin and/or liquid paraffin or other mineral oils can be used.

Formulations suitable for topical administration to the eye also include eye drops wherein the active ingredient is dissolved or suspended in a suitable carrier, especially an

administration may include such further agents as sweeteners, thickeners and flavoring agents.

Prodrugs and compositions of the formula of the present invention may also be presented for the use in the form of veterinary formulations, which may be prepared, for example, by methods that are conventional in the art.

Provided below is a brief summary of cells and target enzymes that are useful to activate the prodrugs of this invention.

Tyrosine Kinases

The tyrosine kinase superfamily comprises the EGF receptor (EGFR), the macrophage colony-stimulating factor (CSF-1) receptor (v-fms), and the insulin receptor, which shows 30 to 40% identity with the product of the ros oncogene. More specifically, the members of this superfamily include v-src, c-src, EGFR, HER2, CSF-1 receptor, c-fms, v-ros, insulin receptor, and c-mos. See Figure 8.5 of Burck, K.B. et al., eds. (1988). Overexpression of members of the type 1 receptor tyrosine kinase superfamily has been documented in many types of cancer (Eccles, S.A. et al. (1994-95)). Overexpression of tyrosine kinases is linked to exposure to the α-cancer biologic agent TNF-α (Hudziak, R.M. et al. (1988) and Hudziak, R.M. et al. (1990)) and to chemotherapy (Stühlinger et al. (1994)).

The transforming gene of the Rous sarcoma virus, v-src, encodes an enzyme that phosphorylates tyrosine residues on proteins. The c-src proto-oncogene is found on chromosome 20. Tissues and cell lines derived from tumors of neuroectodermal origin having a neural phenotype express high levels of c-src accompanied by high specific kinase activity.

Several groups of investigators have reported overexpression of c-erbB-2/neu ("HER2") oncogene in cancer cells. Brison (1993) noted that erbB proto-oncogene is amplified in human tumors with resultant overexpression in most cases. Amplification of the c-erbB-2/neu oncogene has been reported in human mammary tumors (Slamon, et al. (1987), van de Vijver et al. (1987), Pupa et al. (1993), and Andersen et al. (1995)) and in bladder tumors (Sauter et al. (1993)), and in every case amplification was accompanied by overexpression. c-erbB-2/neu overexpression also has been reported in ovarian cancer

DHFR is transfected into NIH 3T3 cells. Candidate drugs are added in varying concentrations and cell killing and inhibition of proliferation are assayed.

Antimetabolites dependent on dihydrofolate reductase activity can be synthesized by the attachment of, for example, an alkylating group to either the N5 or the C6 position of dihydrofolate. Reduction of the N5-C6 bond by DHFR will result in the release of the alkylating agent. In addition to the alkylating groups, any moiety whose release by DHFR results in the production of a toxin or an antimetabolite will be useful in the practice of the invention. These compounds can be further modified by the addition of a phosphatese or phosphoramidate moiety.

Multidrug Resistant Tumors

Multidrug resistance (MDR) is a generic term for the variety of strategies tumor cells use to evade the cytotoxic effects of anticancer drugs. MDR is characterized by a decreased sensitivity of tumor cells not only to the drug employed for chemotherapy but also to a broad spectrum of drugs with neither obvious structural homology nor common targets. This pleiotropic resistance is one of the major obstacles to the successful treatment of tumors. MDR may result from structural or functional changes at the plasma membrane or within the cytoplasm, cellular compartments, or nucleus. Molecular mechanisms of MDR are discussed in terms of modifications in detoxification and DNA repair pathways, changes in cellular sites of drug sequestration, decreases in drug-target affinity, synthesis of specific drug inhibitors within cells, altered or inappropriate targeting of proteins, and accelerated removal or secretion of drugs.

One of the mechanisms implicated in MDR results from amplification and over-expression of a gene known as the ATP-dependent multidrug resistant associated protein (MRP) in drug selected cell lines. For a review of the mechanisms of MDR, see Gottesman, M.M. et al. (1995) and Noder et al. (1996).

To establish MDR cell lines, drug selections are conducted in either a single step or in multiple steps as described in Gottesman, M.M. et al. (1995) and Simon, S.M. and Schindler, M. (1994), and references cited therein. The isolation of DNA sequences coding for MDR from various mammalian species is described in Gros, P. et al. (1986), Gudkov, A.V. et al. (1987), and Roninson, I.B. et al. (1984), and reviewed in Gottesman, M.M. et al. (1995), and cells can be engineered to express varying levels of this enzyme as

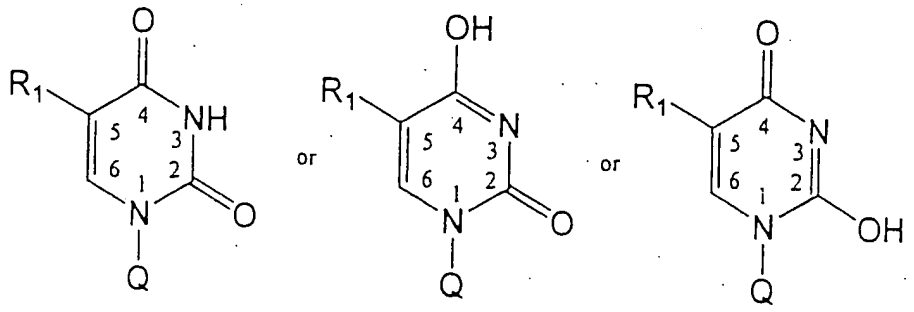
methotrexate, 5-fluorouracil [CMF]) after surgery. This enhanced TS expression is in addition to the basic increase of TS which results from loss of tumor suppressor function. The principal reaction normally performed by TS is the synthesis of deoxythymidine monophosphate (dTMP) and dihydrofolate (DHF) from deoxyuridine monophosphate (dUMP) and N(5),N(10)-methylene-tetrahydrofolate (THF). In one embodiment, a derivative of uracil or THF is provided to cells expressing TS. For purposes of this invention, "uracil" (base only) and "uridine" (base and sugar) are used interchangeably and synonomously. Table 4 (below) summarized the many cancer types impacted by elevated TS expression.

and indicate that tertiary structure also is very well conserved. The sequence alignment of the species of TS whose three dimensional structures have been determined and is shown in Schiffer, C.A. et al. (1995). From these amino acid sequences, the DNA sequences can be deduced or isolated using methods well known to those of skill in the art. Sambrook, et al. (1989). Alternatively, some 29 TS sequences from different organisms have been cloned and deposited into the DNA databases as described in Carreras, C.W. and Santi, D.V. (1995). The sequence of human thymidylate synthase gene, its cloning, expression and purification is provided in Takeishi, K. et al. (1985), Davisson, V.J. et al. (1989) and Davisson, V.J. et al. (1994). Genes encoding the TS protein and containing the necessary regulatory sequences, are constructed using methods well known to those of skill in the art. The gene encoding TS is introduced to target cells by electroporation, transformation or transfection procedures. Sambrook, et al. (1989). Alternatively, the gene is inserted into an appropriate expression vector by methods well known in the art, e.g., as described in Carreras, C.W. and Santi, D.V. (1995), Miller (1992) and Spector et al. (1998). The expression vector inserts the TS gene into the cells. The cells are then grown under conditions which favor the expression and production of TS protein.

Human gastric cancer cell lines, MKN-74, MKN-45, MKN-28 and KATO-III can be used in the assay described above to identify potential therapeutic agents which are selective substrates for TS. MKN-74 and MKN-45 are established from well and poorly differentiated adenocarcinomas, respectively. These cell lines and culture conditions are described in Osaki, M. et al. (1997) and references cited therein. Alternatively, tumor cell lines such as those described by Copur, S. et al. (1995), which have been selected by 5-FU to overexpress thymidylate synthase may be used.

Quantitation of TS can be performed using enzymatic biochemical assays that are well known to those with skill in the art. To quantify the level of TS protein and TS gene expression from human tumor tissue samples, the methods as reported by Johnston, P.G. et al. (1991) and Horikoshi, T. et al. (1992) provide sensitive assays. Alternatively, the PCR method of Lönn, U. et al. (1996) is used to assay TS gene amplification and identify cells that are useful in the method of identifying therapeutic agents as described herein.

As is apparent to one skilled in the art, control cell culture systems without drug and separately with a reference drug such as the compounds exemplified below, also are assayed. A lead compound is one which preferentially kills target cells with about 2-fold and

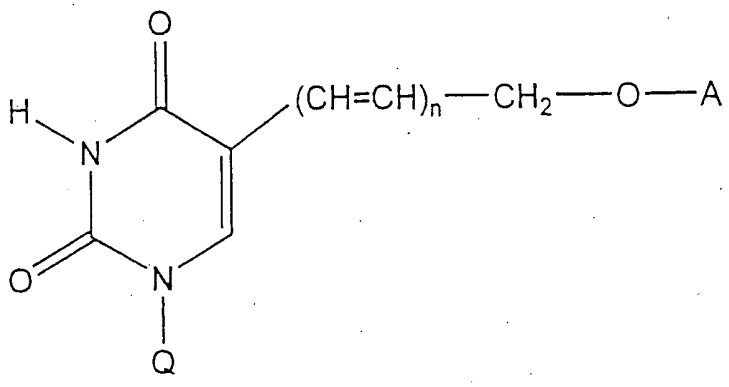


In the above formulae, R_1 (at the 5-position) is or contains a leaving group which is a chemical entity that has a molecular dimension and electrophilicity compatible with extraction from the pyrimidine ring by thymidylate synthase, and which upon release from the pyrimidine ring by thymidylate synthase, has the ability to inhibit the proliferation of the cell or kill the cell.

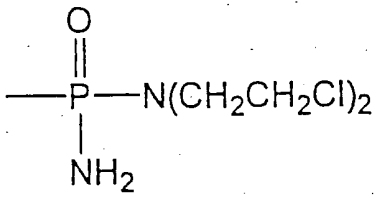
In the above formulae, Q is a phosphate or phosphoramidate derivative containing a chemical entity selected from the group consisting of sugar groups, thio-sugar groups, carbocyclic groups, and derivatives thereof. Examples of sugar groups include, but are not limted to, monosaccharide cyclic sugar groups such as those derived from oxetanes (4-membered ring sugars), furanoses (5-membered ring sugars), and pyranoses (6-membered ring sugars). Examples of furanoses include threo-furanosyl (from threose, a four-carbon sugar); erythro-furanosyl (from erythrose, a four-carbon sugar); ribo-furanosyl (from ribose, a five-carbon sugar); ara-furanosyl (also often referred to as arabino-furanosyl; from arabinose, a five-carbon sugar); xylo-furanosyl (from xylose, a five-carbon sugar); and lyxo-furanosyl (from lyxose, a five-carbon sugar). Examples of sugar group derivatives include "deoxy", "keto", and "dehydro" derivatives as well as substituted derivatives. Examples of thio sugar groups include the sulfur analogs of the above sugar groups, in which the ring oxygen has been replaced with a sulfur atom. Examples of carbocyclic groups include C_4 carbocyclic groups, C_5 carbocyclic groups, and C_6 carbocyclic groups which may further have one or more subsituents, such as -OH groups.

In one embodiment, Q is a β-D-ribofuranosyl group of the formula:

In a still further aspect, the candidate therapeutic agent is a compound of the formula:

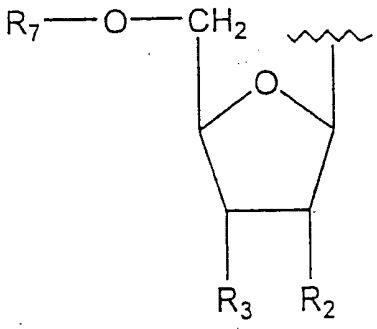


wherein n is an integer from 0 to 10; wherein A is a phosphoramide derivative, or a compound of the formula:



and wherein Q is selected from the group consisting of H, an unsubstituted or substituted sugar as defined above and a substituted or unsubstituted carbocyclic as defined above.

In a further embodiment, the compounds described above, are modified by Q having the structure:



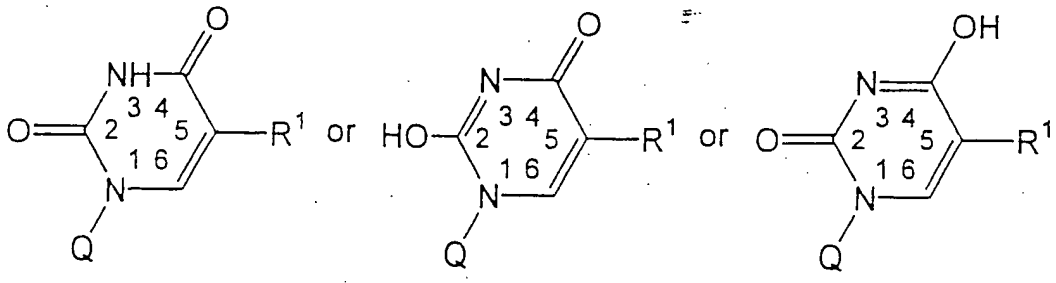
wherein R_7 is selected from the group consisting of H, phosphoryl, phosphoramidate and derivatives thereof, and wherein R_2 and R_3 are the same or different and are independently -H or -OH. In one embodiment, R^7 is not H. For these embodiments, R_1 also can have the structure $-(CH=CH)_n-R_4$, wherein n is an integer from 0 to 10, and R_4 is selected

to mercury but with preferred pharmacological properties could be substituted. For general methods for synthesis of substituted pyrimidines, for example, U.S. Patent Nos. 4,247,544; 4,267,171; and 4,948,882; and Bergstrom et al. (1981). The above methods would also be applicable to the synthesis of derivatives of 5-substituted pyrimidine nucleosides and nucleotides containing sugars other than ribose or 2'-deoxyribose, for example 2'-3'-dideoxyribose, arabinose, furanose, lyxose, pentose, hexose, heptose, and pyranose. An example of a 5-position substituent is the halovinyl group, e.g. E-5-(2-bromovinyl)-2'-deoxyuridylate. Barr, P.J. et al. (1983). This compound is synthesized as follows as described in the experimental section.

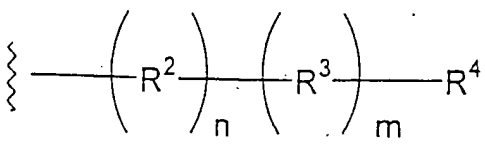
Alternatively, 5-bromodeoxyuridine, 5-iododeoxyuridine, and their monophosphate derivatives are available commercially from Glen Research, Sterling, VA (USA), Sigma-Aldrich Corporation, St. Louis, MO (USA), Moravek Biochemicals, Inc., Brea, CA (USA), ICN, Costa Mesa, CA (USA) and New England Nuclear, Boston, MA (USA). Commercially-available 5-bromodeoxyuridine and 5-iododeoxyuridine can be converted to their monophosphates either chemically or enzymatically, though the action of a kinase enzyme using commercial available reagents from Glen Research, Sterling, VA (USA) and ICN, Costa Mesa, CA (USA). These halogen derivatives could be combined with other substituents to create novel and more potent antimetabolites.

General Synthesis of Compounds of Class II

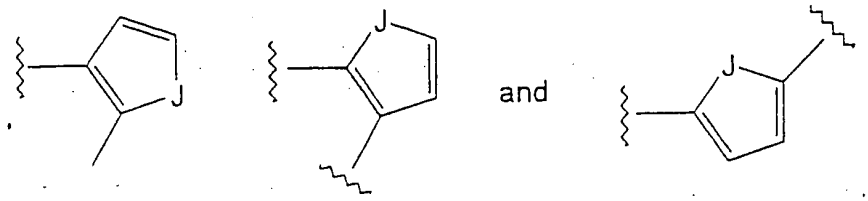
In a further aspect, the prodrug contacted with the cell overexpressing thymidylate synthase is an L or D isomer of a compound of the formula:



In the above formulae, R^1 is a moiety of the formula:

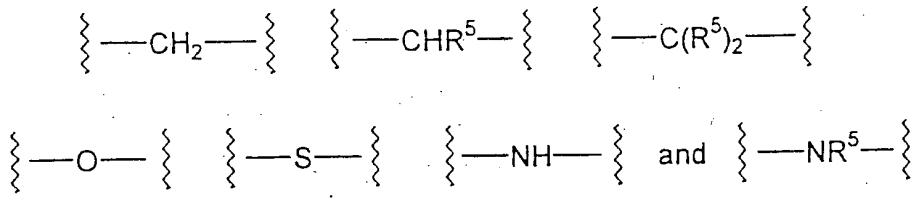


In one embodiment, R^2 is a heteroaromatic group having a structure selected from the group consisting of:



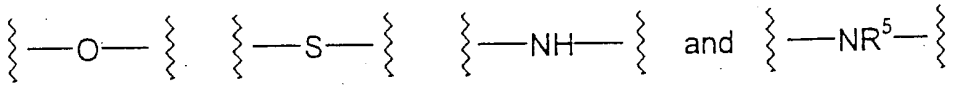
wherein J is a heteroatom, such as -O-, -S-, or -Se-, or a heteroatom group, such as -NH- or $-NR^{ALK}-$, where R^{ALK} is a linear or branched alkyl having 1 to 10 carbon atoms or a cycloalkyl group having 3 to 10 carbon atoms.

In the above formulae, R^3 is a divalent spacer moiety, also referred to as a spacer unit. In one embodiment, R^3 is a divalent spacer moiety having a structure selected from the group consisting of:

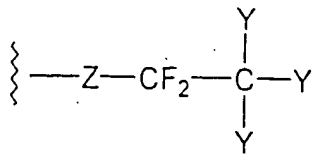


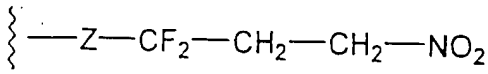
wherein R^5 is the same or different and is independently a linear or branched alkyl group having from 1 to 10 carbon atoms, or a cycloalkyl group having from 3 to 10 carbon atoms.

In one embodiment, R^3 is a divalent spacer moiety having a structure selected from the group consisting of:

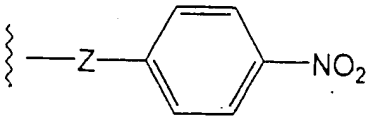


In the above formula, n is an integer from 0 to 10 and, m is 0 or 1. In one embodiment, n is an integer from 0 to 10 and, m is 1. In one embodiment, n is 0 and m is 0. In one embodiment, when R^7 is -H, then n is not zero. In one embodiment, when R^7 is -H, then m is not zero. In one embodiment, when R^7 is -H, then n is not zero and m is not zero. In one embodiment, when R^7 is -H, then R^4 is not a halogen (i.e., -F, -Cl, -Br, -I). In one embodiment, when R^7 is -H, and m is zero, then R^4 is not a halogen (i.e., -F, -Cl, -Br, -I). In one embodiment, when R^7 is -H, and m is zero and n is zero, then R^4 is not a halogen (i.e., -F, -Cl, -Br, -I).



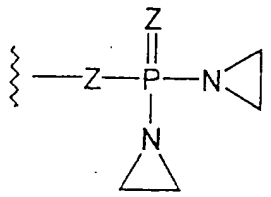


and

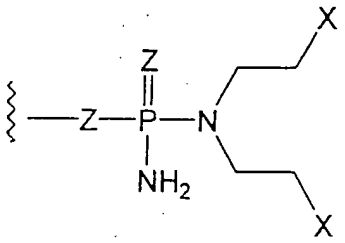


wherein X is -Cl, -Br, -I, or other potent leaving group (including, but not limited to, -CN, -OCN, and -SCN); Y is the same or different, and is independently -H or -F; and Z is the same or different and is independently -O- or -S-.

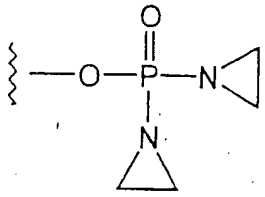
In one embodiment, R^4 is or contains a group having a structure selected from the group consisting of:



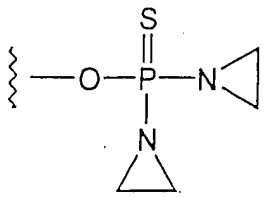
and



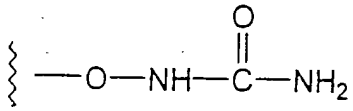
In one embodiment, R^4 is or contains a group having a structure selected from the group consisting of:



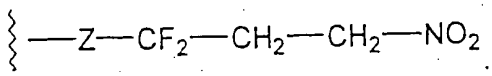
and



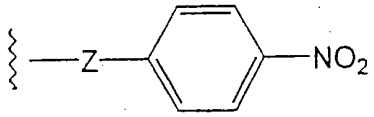
In one embodiment, R^4 is or contains a group having the structure:



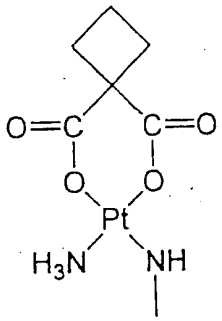
In one embodiment, R^4 is or contains a group having the structure:



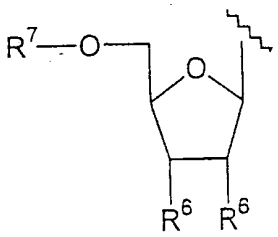
In one embodiment, R^4 is or contains a group having the structure:

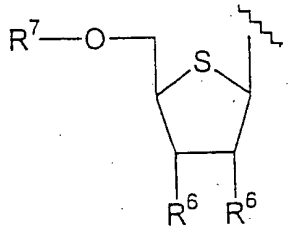


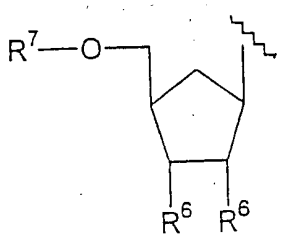
In one embodiment, R^4 is or contains a chemical entity selected from the group consisting of: -Br, -I, -O-alkyl, -O-aryl, O-heteroaryl, -S-alkyl, -S-aryl, -S-heteroaryl, -CN, -OCN, -SCN, $-NH_2$, -NH-alkyl, $-N(alkyl)_2$, -NHCHO, -NHOH, -NHO-alkyl, NH_2CONHO-, $NHNH_2$, $-N_3$, and a derivative of cis-platin, such as:

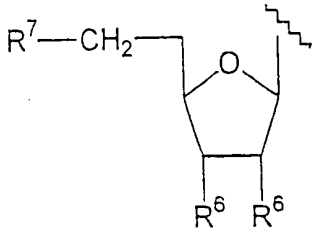


In the above formulae, Q is or contains a sugar moiety or a similar moiety which supports functional binding of the prodrug to the enzyme, *e.g.*, TS or TK. In one embodiment, Q is selected from the group consisting of:

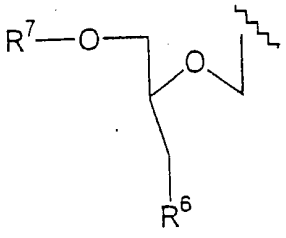




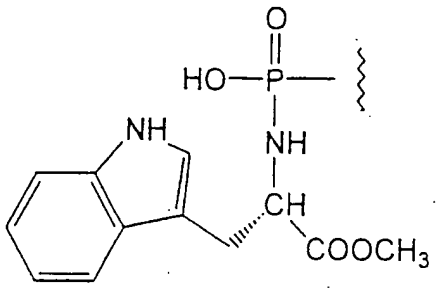




and

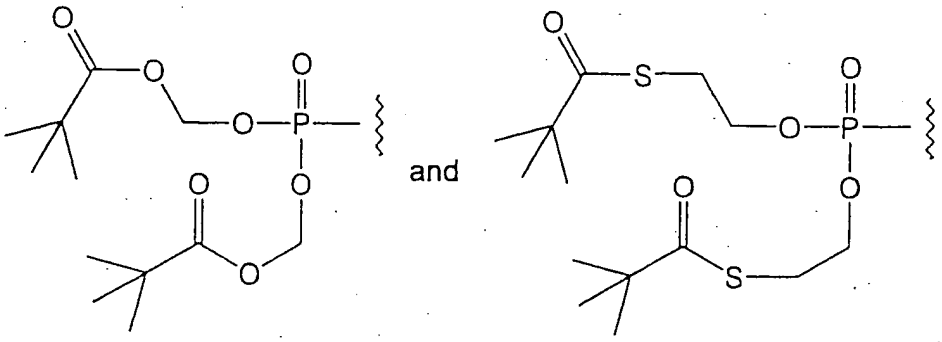


In one embodiment, R^7 is a phosphoramidate group derived from tryptophan. In one embodiment, R^7 is or contains a group having the structure:



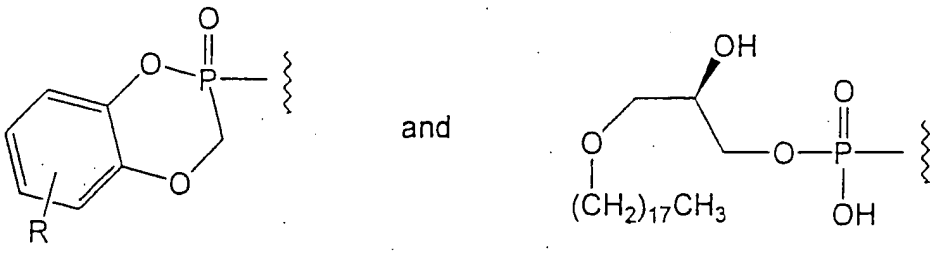
The above group, and methods for its preparation, are described in Abraham et al., (1996).

In one embodiment, R^7 is a phosphate group. In one embodiment, R^7 is or contains a group having a structure selected from the group consisting of:



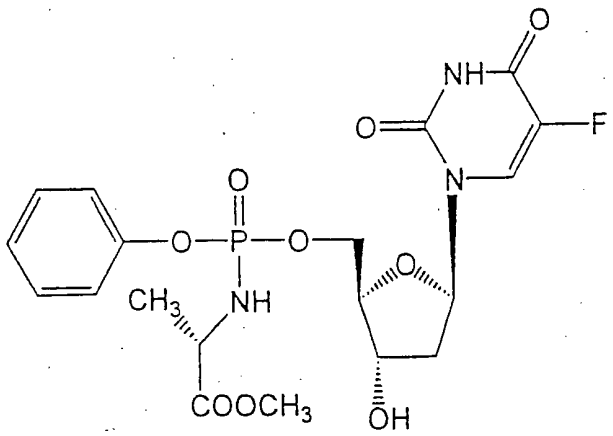
The first of the two above groups, and methods for its preparation, are described in Freed et al. (1989); Sastry et al., (1992); Farquhar et al. (1994), and Farquhar et al. (1995). The second of the two above groups, and methods for its preparation, are described in Valette et al. (1996); and Benzaria et al. (1996).

In one embodiment, R^7 is or contains a group having a structure selected from the group consisting of (where R is an aromatic substituent):

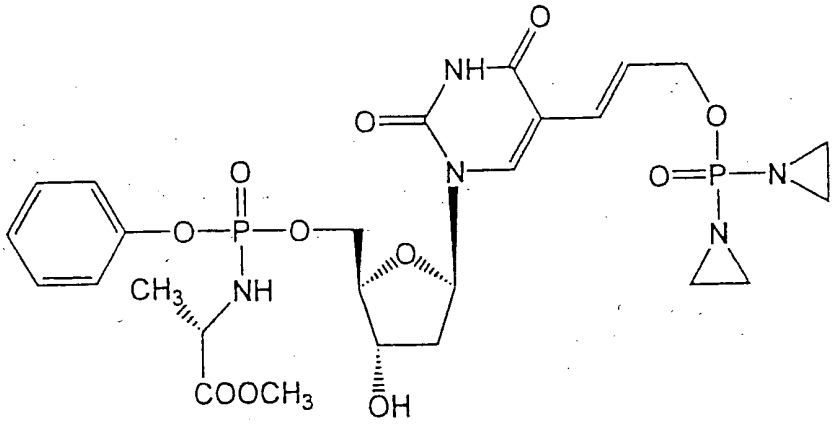


The first of the two above groups, and methods for its preparation, are described in Meier et al. (1997); Meier et al., (1997); and Meier et al., (1997). The second of the two

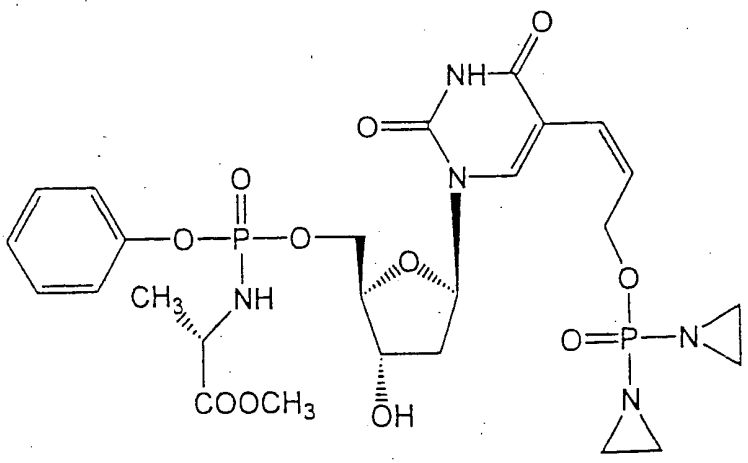
In one embodiment, the prodrug is a compound of the formula:



In one embodiment, the prodrug is a compound of the formula:



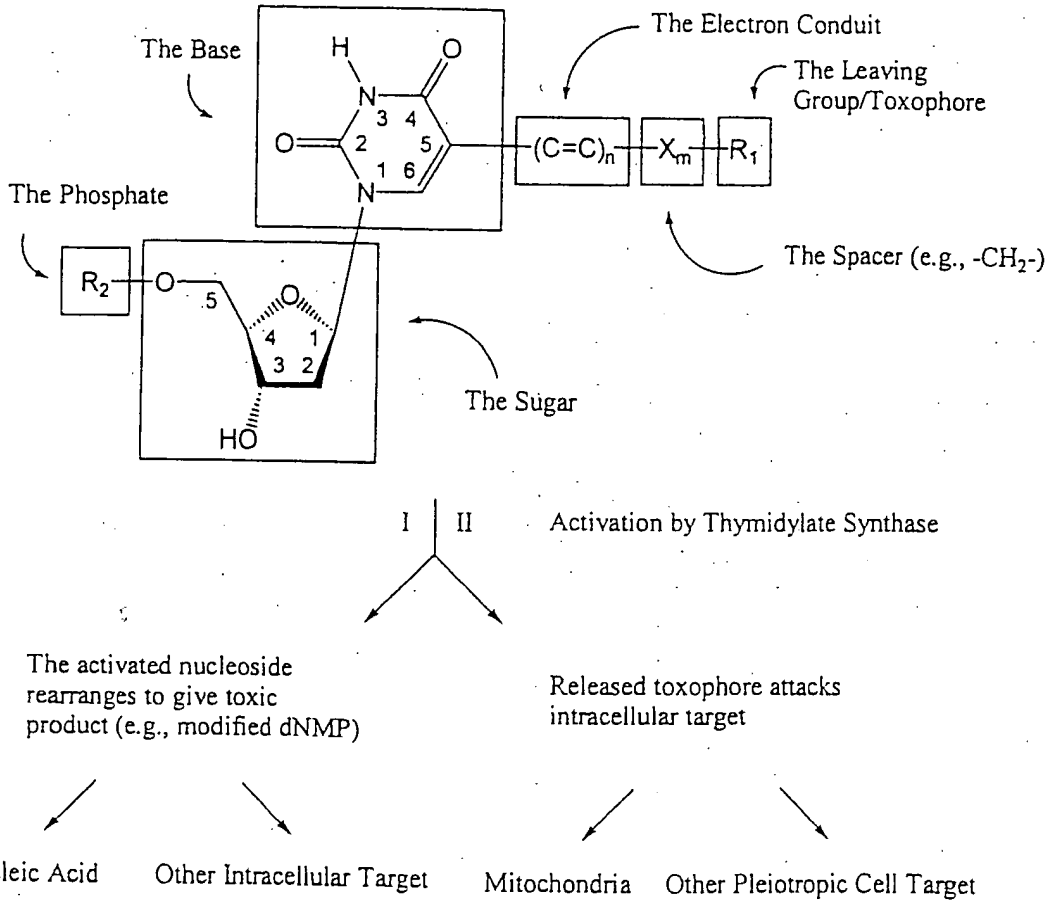
In one embodiment, the prodrug is a compound of the formula:



not depend upon its orientation with respect to dUMP's uracil ring, as is the case with the vinyl and allyl molecules.

Two distinct approaches have been taken to design the nucleotide-based prodrugs of this invention. One is based on the structure of BVDU monophosphate and features a leaving group/toxin directly attached to the terminus of a (poly)vinyl substituent at C5 of dUMP. This is the vinyl tether approach. These compounds are defined in part as "Class I" compounds. The other is based on the structure of TFPe-dUMP and is similar to the first but has a methylene unit separating the leaving group/toxin and the unsaturated unit and thus contains an allyl or propargyl unit, described below as "Class II". This is the allyl tether approach.

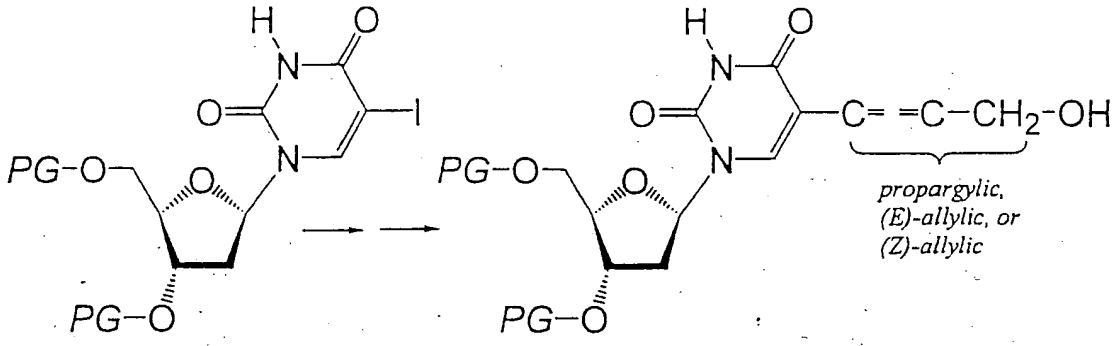
Structures for 5'-phosphoramidate versions of each type are shown below:



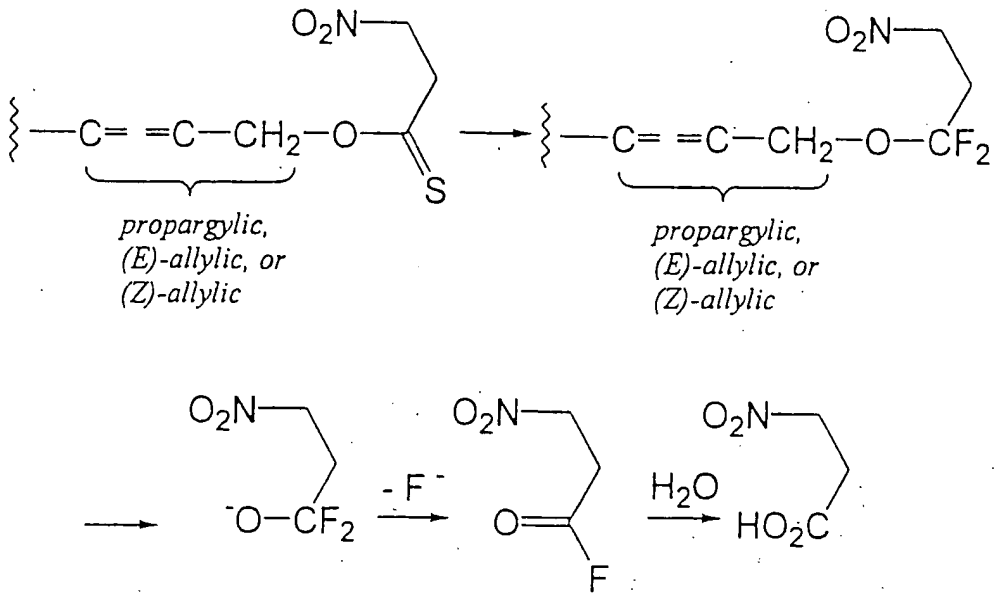
The mechanism of activation of a propargyl version of the allyl tether approach has a precedent in the interaction of both 5-ethynyl-2'-deoxyuridine 5'-monophosphate (EdUMP) and 5-(3-hydroxy-1-propynyl)-2'deoxyuridine 5'-monophosphate (HOPdUMP)

allyl-based ones are best prepared by Heck coupling of an (E)-tributylstannylated ethylene (Crisp (1989)).

Closely following the literature procedures, a t-butyldimethylsilyl propargyl ether-equipped 3', 5'-di-O-protected 2'-deoxyuridine (Graham et al. (1998), De Clercq et al. (1983)) is prepared and a portion of it converted to the corresponding (Z)-allyl ether (Robins and Barr (1983)) is reduced. Because the TBAF-mediated removal of a TBDMS group generates an oxyanion that ran be functionalized *in situ*, these TBDMS-protected propargyl- and (Z)-allytic-tethered nucleosides will serve as convenient precursors to some of the toxophore-equipped targets. For the (E)-allyl alcohol equipped nucleoside, the known O-tetrahydropyranyl ether derivative is prepared by the literature Heck coupling of an (E)-tributylstannylated ethylene (Crisp (1989)).



Using a two step literature protocol (Phelps et al. (1980) Hsiao and Bardos (1981)), the propargylic and (E) and (Z)-allylic alcohols are converted to their corresponding bis-aziridinyl phosphoramidates or thiophosphoramidates so that TS processing of the 5'-mononucleotide versions will release an active metabolite of the cytostatic drugs TEPA or ThioTEPA (Dirven et al. (1995)), respectively.



p-Nitrophenyl ether derivatives of the CS propargylic and (E) and (Z)-allylic alcohol-equipped 2'-deoxyuridines provide good reagents for *in vitro* TS enzyme assays, because the spectrophotometric, kinetic determination of released p-nitrophenol will identify those tethered dUMP platforms that bind to TS in a manner that permits facile catalytic release of a leaving group from the end of the tether. The needed p-nitrophenyl ethers will be obtained either directly from the alcohols by a base-catalyzed condensation with 4-fluoronitrobenzene or *de novo* by a Heck coupling of an appropriate p-nitrophenyl propargyl or allyl ether. The 5'-monophosphates needed for the TS assay are generated from the nucleosides either enzymatically or chemically according to a well-established regioselective 5'-monophosphorylation protocol (Imai et al. (1969)).

overexpression of thymidine kinase in most tumor cells as a result of tumor suppressor gene loss (Hengstschlager et al. (1996)). Similarly, preferential activation of the phosphorylated prodrug or phosphoramidate derivative will occur in tumor cells because of the overexpression of thymidylate synthase accompanying tumor suppressor loss (Li, W. et al. (1995)) and chemotherapy (Peters, G.J. et al. (1995)). Combinatorial chemistry targeting the 5-position of the uridine ring (from which the cell toxin may be generated), or the 5'-position of the pentose sugar (to facilitate binding to thymidylate synthase), will greatly expedite the discovery of lead compounds by optimizing the structure of the leaving group (cell toxin) from the uridine ring, as well as facilitating optimization of a phosphorylation competent group at the 5'-position of the pentose sugar. For purposes of the screen shown in Figures 3, 4 and 5, the chemical entity being tested will be generated by either a single site directed chemistry (Figure 4), or simultaneously at two sites on the molecule (Figure 5). Together with the screen shown in Figure 3, a powerful system for discovery of prodrugs targeting thymidylate synthase has been developed. The combinatorial chemistry approach used will be similar to that described in Lam, K.S. (1997). In one embodiment, prodrugs that provide toxic leaving groups are shown in Figure 6. The uridine substrates shown in Figure 6 take advantage of phosphoramidase (present in all cells) and elevated thymidylate synthase (TS) in tumor cells. As is understood by those of skill in the art, this rationalized drug design can be broadly applied to the synthesis of other prodrugs as defined herein.

It should be understood by those skilled in the art that the screen shown in Figure 3 can be applied broadly for the discovery of antibiotics. For example, thymidylate synthase from yeast could be substituted for that of *E. coli* in Figure 4. This would allow the discovery of specific antifungal antibiotics targeting yeast related pathogens. In addition, other enzymes can be subjected to this treatment. For example, prodrugs which target specifically the dihydrofolate reductase activity of infectious agents, like *Pneumocystis carnii*, could be selected. These agents will be selected for specificity for the target enzyme, and can be shown not to activate the enzyme of the natural host by employing the screening assay described in Figure 3. The control cellular constructs would contain the corresponding normal human enzyme, in order to show lack of toxicity when only the normal human enzyme is present.

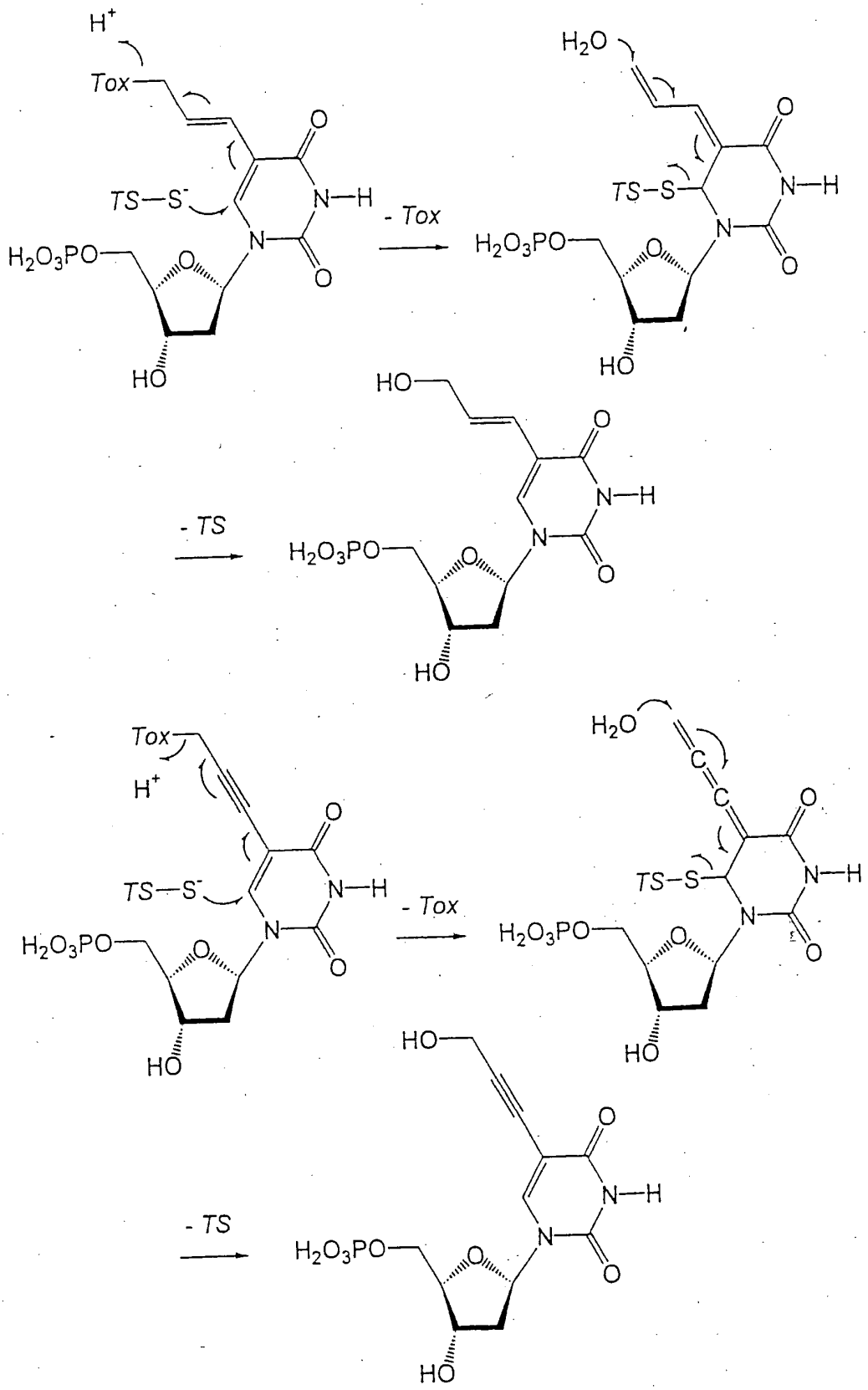
phenylproprionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate and undecanoate. Other examples of salts include anions of the compounds of the present invention compounded with a suitable cation such as Na^+, NH_4^+, and NW_4^+ (wherein W is a C_{1-4} alkyl group).

For therapeutic use, salts of the compounds of the present invention will be pharmaceutically acceptable. However, salts of acids and bases which are non-pharmaceutically acceptable may also find use, for example, in the preparation or purification of a pharmaceutically acceptable compound.

Esters of the prodrugs or compounds identified by the method of this invention include carboxylic acid esters (i.e., -O-C(=O)R) obtained by esterification of the 2'-, 3'- and/or 5'-hydroxy groups, in which R is selected from (1) straight or branched chain alkyl (for example, n-propyl, t-butyl, or n-butyl), alkoxyalkyl (for example, methoxymethyl), aralkyl (for example, benzyl), aryloxyalkyl (for example, phenoxymethyl), aryl (for example, phenyl optionally substituted by, for example, halogen, C_{1-4}alkyl, or C_{1-4}alkoxy or amino); (2) sulfonate esters, such as alkylsulfonyl (for example, methanesulfonyl) or aralkylsulfonyl; (3) amino acid esters (for example, L-valyl or L-isoleucyl); (4) phosphonate esters and (5) mono-, di- or triphosphate esters. The phosphate esters may be further esterified by, for example, a C_{1-20} alcohol or reactive derivative thereof, or by a 2,3-di-(C_{6-24})acyl glycerol. In such esters, unless otherwise specified, any alkyl moiety present advantageously contains from 1 to 18 carbon atoms, particularly from 1 to 6 carbon atoms, more particularly from 1 to 4 carbon atoms. Any cycloalkyl moiety present in such esters advantageously contains from 3 to 6 carbon atoms. Any aryl moiety present in such esters advantageously comprises a phenyl group. Examples of lyxo-furanosyl prodrug derivatives of the present invention include, for example, those with chemically protected hydroxyl groups (e.g., with O-acetyl groups), such as 2'-*O*-acetyl-lyxo-furanosyl; 3'-*O*-acetyl-lyxo-furanosyl; 5'-*O*-acetyl-lyxo-furanosyl; 2',3'-di-*O*-acetyl-lyxo-furanosyl and 2',3',5'-tri-*O*-acetyl-lyxo-furanosyl.

Ethers of the compounds of the present invention include methyl, ethyl, propyl, butyl, isobutyl, and sec-butyl ethers.

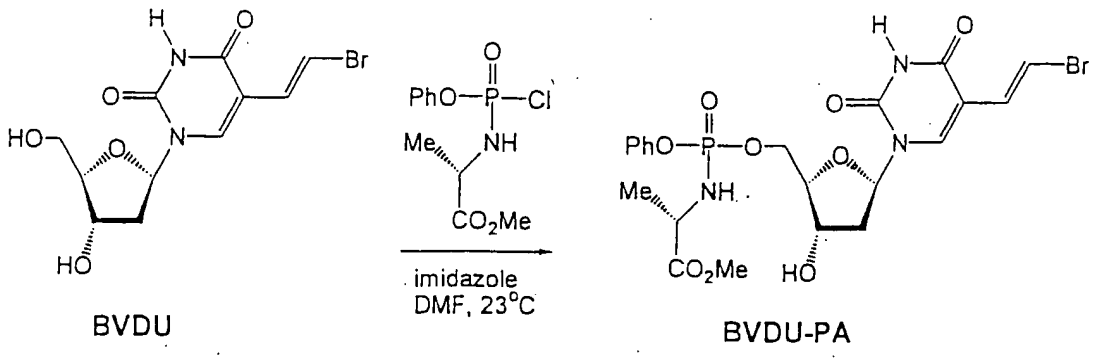
In a further embodiment, the substrate may not be chemically related to pyrimidines or folates, but rather synthesized based upon known parameters of rational drug design. See Dunn, W.J. et al. (1996).


H+
Tox
TS—S-
H2O3PO
HO
- Tox
H2O
- TS
Tox
H+
TS—S-
H2O3PO
HO
- Tox
H2O
- TS
H2O3PO
HO

(pseudo-t, 1, H1'), 6.08 (t, exchanges with D_2O, 1, alaninyl NH), 5.45 (bs, exchanges with D_2O, 1, 3'OH), 4.32 (m, 1, H4'), 4.22 (m, 2, 5'CH_2), 3.97 (m, 1, H3'), 3.86 (t, 1, alaninyl CH), 3.58 (s, 3, CO_2Me), 2.15 (m, 2, 2'CH_2), 1.23 (t, 3, alaninyl CH_3). $J_{\text{vinyl CH-vinyl CH}} = 13.5$, $J_{H1'-H2'} \sim 6.8$, $J_{H2'-H3'} \sim 5$, $J_{H3'-H4'} \sim 0$, alaCH-Ala-NH_3 NH ~ 6 Hz. $^1H/^1H$ COSY 2D NMR spectroscopy provided confirmation of spectral assignments.

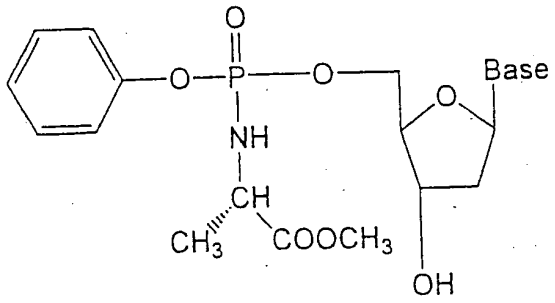
In a similar fashion, 5-Fluoro-2'-deoxy-5'-uridyl phenyl L-alaninylphosphoramidate (5FUdR-PA), was obtained in a purity of at least 98%, by TLC and ^1H NMR. ^1H NMR ($(CD_3)_2SO$) δ 11.9 (bs, exchanges with D_2O, 1, N3H), 7.88 (t, 1, H6), 7.36 (m, 2, Ph), 7.19 (m, 3, Ph), 6.15 (pseudo-t, 1, H1'), 6.07 (t, exchanges with D_2O, 1, alaninyl NH), 5.42 (bs, exchanges with D_2O, 1, 3'OH), 4.21 (m, 3, H4' and 5'CH2), 3.98 (m, 1, H3'), 3.84 (t, 1, alaninyl CH), 3.58 (s, 3, CO_2Me), 2.08 (m, 2, 2'CH_2), 1.22 (t, 3, alaninyl CH3). $J_{H6-5F} = 7.1$, $J_{H1'-H2'} \sim 5.2$, $J_{H2'-H3'} \sim 2$, $J_{H3'-H4'} \sim 0$, $J_{\text{alaninyl CH-alaninyl NH}} \sim 6$ Hz. $^1H/^1H$ COSY 2D NMR spectroscopy provided confirmation of spectral assignments. Low-resolution mass spectrum (DCI-NH3), m/z 505 (MNH_4+), 488 (MH+).

It was reasoned that the direct condensation of an unprotected 2'-deoxyribonucleoside with PMPC might very well proceed to give the desired phosphoramidate and in a 5'-O-regioselective manner if it were conducted in the absence of base but the presence of a scavenger for the HCl produced. Both BVDU and 5FUdR condensed with the McGuigan reagent in the presence of imidazole in anhydrous DMF solution to give the desired BVDU-PA and 5FUdR-PA, respectively (see below). The reaction conditions have not been optimized, and although the reactions do not proceed to completion, the readily separable product mixture in both cases consists largely of only desired product and starting material, by thin layer chromatography (TLC). The synthetic scheme is summarized below.

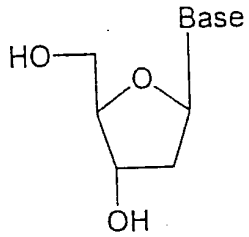


BVDU BVDU-PA

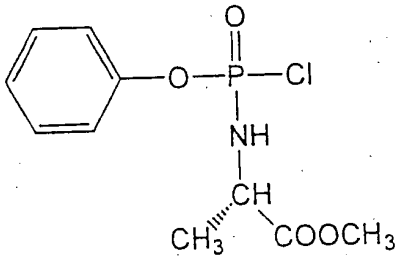
L-methoxyalanine phosphorochloridate. Thus, in a further embodiment, the present invention pertains to a method of forming a compound of the formula:



wherein "Base" denotes a nucleic acid base (such as uracil, thymine, cytosine, adenine, or guanine, but preferably uracil, or a derivative thereof); which method comprises the step of reacting a compound of the formula:



with a compound of the formula:



in the presence of an HCl scavenger.

In one embodiment, the furanosyl nucleoside is a uridine nucleoside. In another embodiment, the furanosyl nucleoside is a uridine ribofuranosyl nucleoside. In another embodiment, the furanosyl nucleoside is a uridine β-D-ribofuranosyl nucleoside. Thus, in one embodiment, the present invention pertains to a method of forming a compound of the formula:

Chemical and Cell-Based Assays

Two cell lines, H630R10 (Copur, et al. (1995)) and normal colon epithelial cells, CCD18co (ATCC) were used in these assays. The H630P (Copur et al. (1995)) cell line was selected for resistance to 10μM 5-FU to give rise to H630R10. The characterization of these cell lines which expresses an elevated level of the TS enzyme. A normal colon epithelial cell type CCD18co (available from the ATCC) was used for comparison with H630R10 for sensitivity to test compounds. Cell lines expressing p185 - HER2 or neomycin marker alone were prepared as described by Pegram et al. (1997) shows that H630R and H630R10 expresses 10 fold increased thymidylate synthase enzyme as compared with CCD18co as determined by Western blot analysis.

The ability of the test compounds to block proliferation of cells was determined by the crystal violet procedure (Sugarman et al. (1985) and Antelman et al. (1995)).

Compounds were dissolved in dimethyl sulfoxide to a concentration of 1M. They were further diluted as necessary into DMEM cell culture medium, and subsequently into the first wells of the 96 Well microtiter plate. Each concentration was tested in triplicate on the target cell line. Compound concentrations from 1 μM to 3000 μM were tested. Cells were incubated with compound for 72 hours, the plates were washed, and the cells fixed with methanol and stained with crystal violet as described in Sugarman et al. (1995) and Antelman, et al. (1995).

Western Blot Analysis of TS Levels in Cell Lines

Western blot experiments were performed with the human normal colon epithelium cell type CCD18co (obtained from ATCC, Manassas, VA), colon adenocarcinoma cell line H630R10 (obtained from Dr. S. Copur, Yale University), and HER2-transfected breast cancer cell lines (Pegram et al. (1997)). Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5,150 mM NaCl, 0.5% Triton X-100, 0.1% SDS and 0.5% Deoxycholic acid, sodium salt and protease inhibitors). Protein concentrations were determined by using BCA-200 protein assay kit (obtained from Pierce, Rockford, IL). 10 μg of proteins from each cell line were resolved by 12% SDS-PAGE. The separated proteins were transferred onto PVDF membrane (obtained from Amersham, England), followed by immunoblot with human thymidylate synthase monoclonal primary antibody and anti-tubulin monoclonal antibody (manufactured by NeoMarkers, Fremont, CA). Horseradish peroxidase linked

5 μL of PCR products were resolved by electrophoresis in 2% agarose gel, followed by staining with SYBR Gold nucleic acid gel stain (obtained from Molecular Probes, Eugene, OR). The DNA bands corresponding to thymidylate synthase were quantified and normalized to that of β-actin by Molecular Dynamics Storm.

Northern Blot Analysis

Northern blots were obtained from Invitrogen (Carlsbad, CA) and hybridized to a cloned TS cDNA probe. Hybridization signals were normalized vs. ribosomal protein S9 as a housekeeping transcript. A tumor was considered to overexpress TS mRNA if the normalized signal was enhanced at least 2-fold as compared to the normal tissue control.

Cloning, Expression and Purification of Human Thymidylate Synthase

An *E. coli* plasmid expression vector for human thymidylate synthase (TS) was constructed using a modified human TS cDNA in pGCHTS. This clone, obtained from Dr. Dan Santi, contains the complete open reading frame for human TS.

For expression in *E. coli* the complete human TS ORF was subcloned into the T7 promoter expression vector pET28a (Novagen Inc.). The resulting plasmid vector encodes the synthesis of human TS as a recombinant fusion protein with a six histidine followed by a thrombin cleavage site. This fusion protein adds a total of 20 extra amino acids to the amino terminus of human TS. To produce recombinant protein, the human TS expression vector was introduced into the *E. coli* strain BL21(DE3), a strain with the T7 RNA polymerase gene inserted into the chromosome under the control of the lac operator.

The human TS-poly-His fusion protein was purified using a metal chelating affinity resin (Novagen Inc.). Protein purification was followed by electrophoresis on 10% SDS polyacrylamide gels. Protein concentrations were determined using the Pierce BCA protein assay. Approximately 10 mg of human TS fusion protein was recovered from a 500 ml culture of *E. coli*. When visualized with silver stain only the band corresponding to human TS was apparent. The identity of the human TS band was confirmed by performing a Western blot with the anti-TS monoclonal antibody TS106 (NeoMarkers).

human ovarian carcinoma cells. The MCF7/HER2 and MCF7/neo cell lines are target cells for assays of this invention because induction of TS is most pronounced (about 20-fold) in these cell types.

The fluoropyrimidine, 5-FUdR, inhibits cell growth via mechanisms similar to 5-FU. This includes cytotoxic effects resulting from incorporation into nucleic acid, as well as inhibition of TS enzyme activity (Goodman and Gilman (1996)). It is therefore expected that cells expressing higher levels of TS will often be more resistant (have a higher IC_{50}) than cells which express lower amounts of the enzyme. Table 6 (Line 1) demonstrates that this is the result obtained. Furthermore, as expected from earlier work, BVDU has little activity in the assay (Table 6; line 2). This presumably results from the inability of BVDU to be monophosphorylated by human thymidine kinase, a requirement for binding to thymidylate synthase (Balzarini et al. (1987), Balzarini et al. (1993), Carreras and Santi (1995)). There is no significant difference in sensitivity of the two cell lines to BVDU. Activation of BVDU occurs with phosphoramidation of the 5'-hydroxyl of the ribose moiety. BVDU-PA (Table 6, line 3) has more than 10-fold greater activity on the higher expressing H630R10 cell line than on CCD18co cells.

Table 6
Higher Levels of TS Expression Predict Greater Sensitivity to BVDU-PA

	$IC_{50}(\mu M)$[1]	
Compound	CCD18co	H630R10
1. 5-FUdR	9.5M ± 0.42	169.8 ± 2.4
2. BVDU	2540 ± 58.2	2876.2 ± 54.5
3. BVDU-PA	2810.2 ± 75.1	216.7 ± 15.7

[1] Standard error less than 20%

These two cell lines were then compared with respect to sensitivity to 5FUdR, and the phosphoramidate derivative of BVDU (BVDU-PA). Because 5FUdR inhibits cell growth via the same mechanism as 5FU, it is expected that CCD18co will be more sensitive (have a lower IC-50) than H630R10, since the latter cell line has a higher intracellular level of thymidylate synthase. The data presented in Table 7 demonstrated that this is the result obtained, using the crystal violet assay, described above. This result

An additional assay of this invention requires candidate drugs to be screened in reaction mixtures containing human thymidylate synthase with and without N5N10-methylenetetrahydrofolate, and the candidate prodrug. The leaving group of the candidate prodrug (e.g., at the pyrimidine 5 position) is labeled, for example, with tritium using methods well known in the art. The control substrate is similarly labeled (e.g. 5-^{3}H) dUMP, under the same reaction conditions. The assays are done similarly to the description provided in Carreras, C.W. and Santi, D.V. (1995), and references cited therein. The human thymidine synthase can be purified from *E. coli* containing the expressed human thymidylate synthase. See Davisson, V.J. et al. (1989) and Davisson, V.J. et al. (1994). This approach provides a scaleable assay capable of screening large numbers of candidate compounds.

Determination of Intracellular Products of TS Prodrug Metabolism

It is important to determine the intracellular products of TS prodrug metabolism in order to substantiate proposed mechanism of activation and action and to define agents that are candidate thereapeutics. One acceptable view of intracellular metabolism of aryl phosphodiester amidates involves enzymatic conversion into a carboxylic acid, intramolecular rearrangement of the phosphomonoester amidate into to a 5'-monophosphoryl nucleoside (Valette et al. (1996)). However, this mechanism is unlikely to describe intracellular processing of all phosphoramidate-based pronucleotides. For example, a different mechanism was proposed for aryl phosphomonoester amidate processing, one involving the simple direct conversion of the phosphoramidate to the monophosphate species by a phosphoramidate hydrolates (McIntee et al. (1997) and Fries et al. (1995)). Regardless of the mechanism for unmasking the nucleoside monophosphate, this assay will detect products of TS conversion of the intracellular monophosphate to cytotoxic compounds within the cell.

The proposed reaction products of prodrug compounds with TS are shown in Figure 8. To accomplish this task, cells are incubated with an amount of prodrug compounds that induces 50% growth inhibition of high TS expressing cell lines (in the 72H assay supra.). Both low and high TS expresser cells are used (eg., CCD18co vs. H630R10). Time course studies are performed in which treated cells are processed according to the method described by McIntee et al. (1997). Cells are lysed with 60%

The growth properties of this xenograft model are so uniform that a computer model, which can predict the growth trajectory of these tumors following treatment with different drug combinations, has been developed by Dr. Angela Lopez and Dr. Elliot Landau in the Department of Biomathematics at the UCLA School of Medicine (Lopez, et al. pending submission). Because of uniformity and reproducibility of this model, it has become very useful in prectinical testing of novel experimental therapeutics. For example, this model formed the basis for the preliminary *in vivo* analysis of the drug Herceptin® which has recently been approved by the US Food and Drug Administration for the treatment of metastatic breast cancer (Pegram, et al. (1998)). The colon tumor cell lines (HT1080/NEO and HT1080/E2F1.1) have demonstrated tumorigenicity (Banerjee et al. (1998)).

Ras-transformed NIH 3T3 cell lines are transplanted subcutaneously into immunodeficient mice. Initial therapy may be direct intratumoral injection. The expected result is that increased level of expression of human TS or a target enzyme leads to enhanced antitumor activity by the drug candidates. Similar studies are performed with human tumors expressing increasing levels of human TS or a target enzyme, and demonstrating that efficacy in response to drug correlates with their level of human TS expression or target enzyme. Optionally, experiments are be performed as above except the drug will be administered intravenously into the animals to address issues related to efficacy, toxicity and pharmacobiology of the drug candidates. In a further embodiment, control animals will receive an effective amount of a compound identified above under the section entitled "Prodrugs," *supra*.

More specifically, the in vivo studies can be conducted by using two different xenograft models (See Table 9).

For these efficacy experiments prodrug dosing will commence when the means xenograft volumes in each group reach 5OMM^3. Mean tumor volumes of prodrug-treated relative to control-treated animals will be plotted using descriptive statistics with graphical analysis. Statistical tests (single factor ANOVA) will be applied to compare xenograft volume differences between treatment groups. Log-transformation will be applied for ANOVA computations, when indicated, to stabilize variance in xenograft volume data sets. Non-parametric statistical tests can be applied if necessary depending on xenograft volume data distribution. Differences in efficacy between xenograft with high TS expression will be compared to those with low TS expression using prodrug-treated/control-treated tumor volume ratios (T/C ratios) using statistics as described (Pegram, et al. (1997)). This methodology can accommodate differences in intrinsic growth rates between high TS-expressing xenografts and low TS-expressing xenografts.

In vivo studies also can be conducted as described by Harris, MP et al. (1996) and Antelman, D. et al. (1995).

3. Dose Finding Studies in Non-Tumor-Bearing Athymic Mice to Define the Maximum Tolerated Dose (MTD)

Groups of 6 CD-1 nu/nu athymic mice (Charles River Laboratories) (3 male, 3 female) are injected with a single dose of prodrug via the intraperitoneal (I.P.) route. Dosing will begin empirically with the initial dose defined by the amount of a prodrug to achieve a serum concentration equal to the IC_{50} *in vitro*, assuming the volume of distribution of the prodrug to be restricted to the total body water (TBW) compartment of the mouse (0.6 X body mass in grams TBW_{mouse}). If no toxicity is observed at this level, then dose escalation proceeds in groups of 6 mice at half-log intervals. If toxicity is encountered at the empiric dose level, then repeat dose escalation experiments beginning at 10% of the toxic dose will proceed likewise. Mice are observed daily for mobility, grooming behavior, and ability to take food and water. Mouse weights will be assessed weekly. The MTD will be defined as the dose resulting in 10% or less loss in body weight during the observation period, or a dose = 90% of the LD_{10}. If lethality is encountered at a particular dose level then repeat experiment (assuming no toxicity is observed at this level). The observation period is about 60 days. Dose escalation will proceed in

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U.S. Patent No. 4,267,171, Bergstrom, D.E. et al. "C-5 Substituted Cytosine Nucleosides" issued May 12, 1981

CLAIMS

What is claimed is:

1. A method for identifying potential therapeutic agents, comprising:

(a) contacting a target cell with a candidate therapeutic phosphoryl or phosphoramidate prodrug that is a selective substrate for a target enzyme, under conditions that favor the incorporation of the agent into the intracellular compartment of the target cell;

(b) assaying the target cell for inhibition of cellular proliferation or cell killing.

2. The method of claim 1, wherein the prodrug is a phosphoramidate derivative of 2'-deoxyuridine.

3. A method for identifying potential therapeutic agents, comprising:

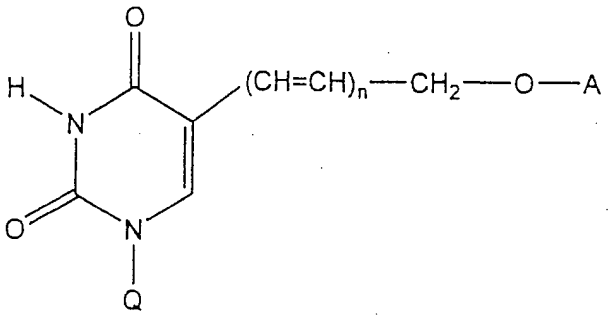
(a) contacting a target cell with a candidate therapeutic phosphoryl or phophoramidate prodrug having a detectably labeled toxic leaving group and that is a selective substrate for a target enzyme, under conditions that favor the incorporation of the agent into the intracellular compartment of the target cell;

(b) assaying the culture media for the amount of label released and comparing it to the amount of label released.

4. The method of claim 3, wherein the prodrug is a phosphoramidate derivative of 2'-deoxyuridine.

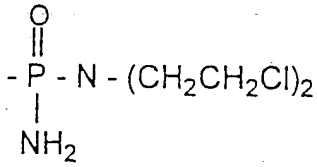
5. The method of any of claims 1 to 5, wherein the target cell is characterized as resistant to a chemotherapeutic drug.

6. The method of any of claims 1 to 5, wherein the target enzyme is amplified as a result of selection *in vivo* by chemotherapy.



;

wherein n is an integer from 0 to 10; wherein A is a phosphoryl or phosphoramide derivative, or a compound of the formula:

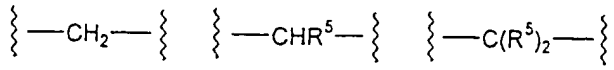


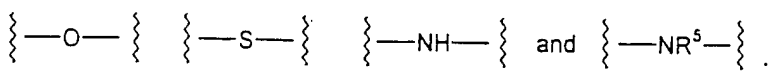
wherein Q is a phosphoryl or phosphoramidate derivative containing a chemical entity selected from the group consisting of sugar groups, thio-sugar groups, carbocyclic groups, and derivatives thereof.

an aromatic hydrocarbyl group comprising one or more unsaturated hydrocarbyl groups; and,

a heteroaromatic group comprising one or more unsaturated hydrocarbyl groups;

R^3 is a divalent spacer moiety selected from the group consisting of:



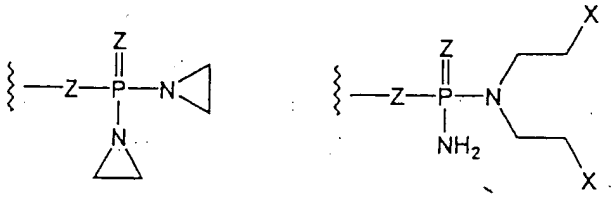


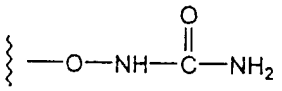
R^5 may be the same or different and is independently a linear or branched alkyl group having from 1 to 10 carbon atoms, or a cycloalkyl group having from 3 to 10 carbon atoms;

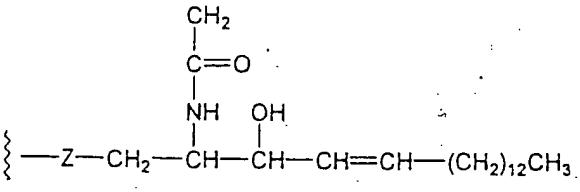
n is an integer from 0 to 10;

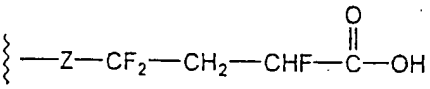
m is 0 or 1;

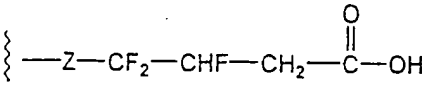
R^4 is a toxophore moiety selected from the group consisting of:

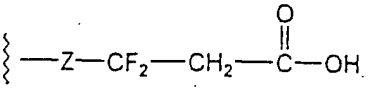


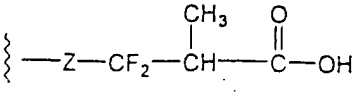




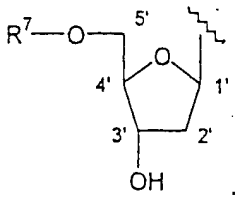




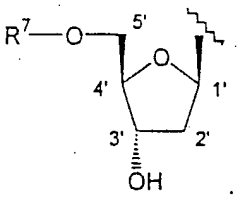




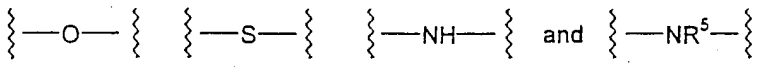
16. A compound according to claim 15, wherein Q is:



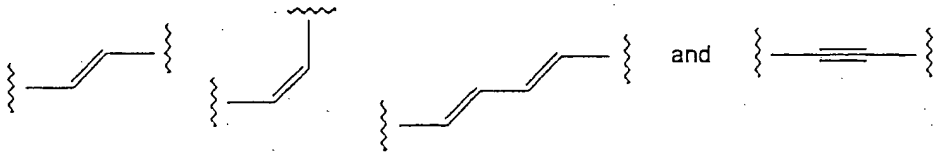
17. A compound according to claim 15, wherein Q is:



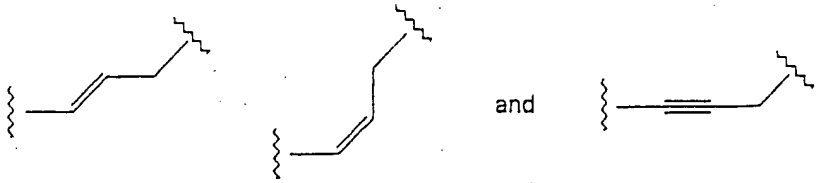
18. A compound according to any one of claims 15 to 17, wherein R^3 is a divalent spacer moiety selected from the group consisting of:



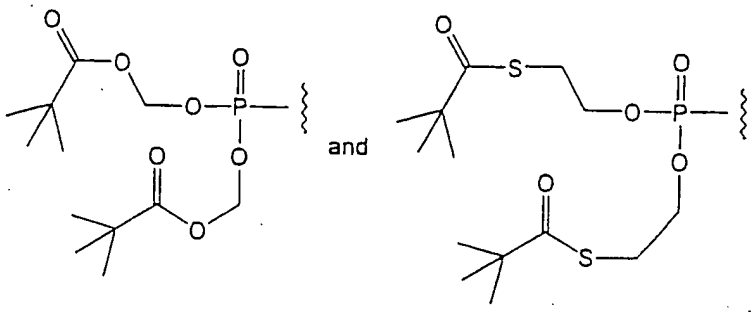
19. A compound according to any one of claims 15 to 17, wherein R^2 is an unsaturated hydrocarbyl group selected from the group consisting of:



20. A compound according to any one of claims 15 to 17, wherein R^2 and R^3, taken together form a structure selected from the group consisting of:

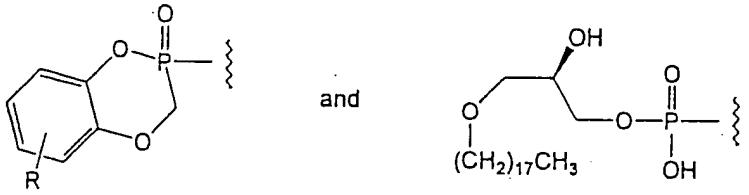


25. A compound according to any one of claims 15 to 22, wherein R^7 is selected from the group consisting of:



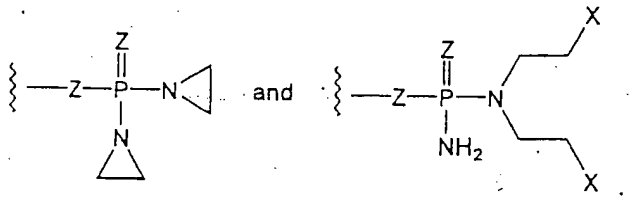
and

26. A compound according to any one of claims 15 to 22, wherein R^7 is selected from the group consisting of:



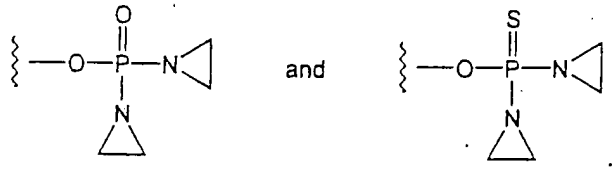
and

27. A compound according to any one of claims 15 to 22, wherein R^4 is selected from the group consisting of:



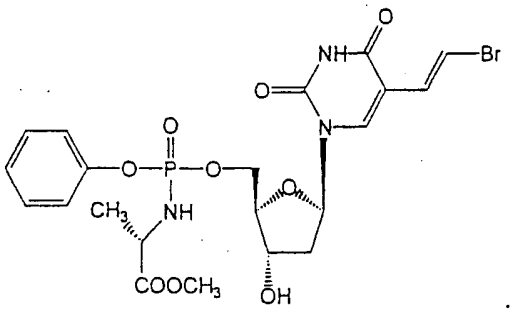
and

28. A compound according to any one of claims 15 to 27, wherein R^4 is selected from the group consisting of:

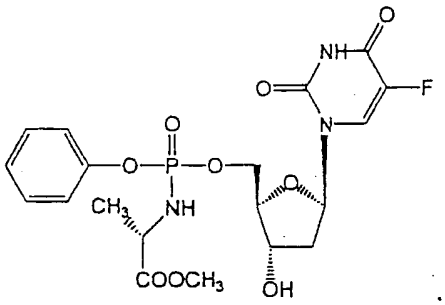


and

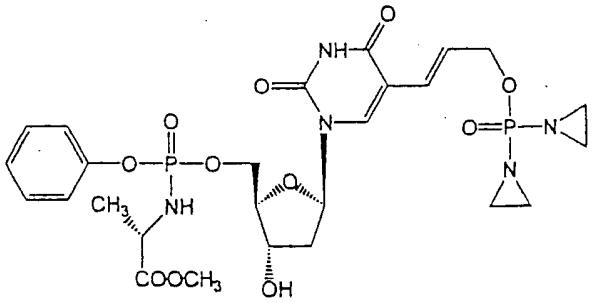
35. A compound of the formula:



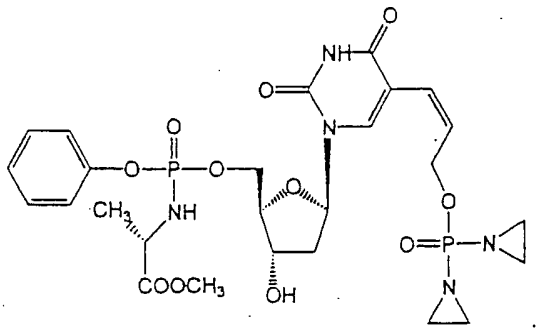
36. A compound of the formula:



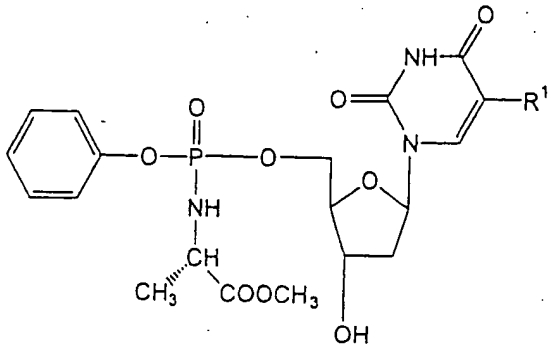
37. A compound of the formula:



38. A compound of the formula:

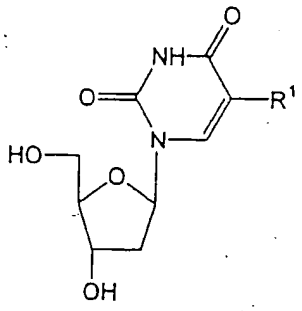


41. A method of forming a compound of the formula:

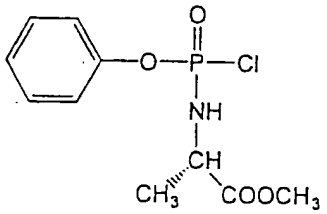


wherein R^1 is a substituent;

which method comprises the step of reacting a compound of the formula:



with a compound of the formula:



in the presence of an HCl scavenger.

42. The method according to claim 40 or 41, wherein said HCl scavenger is imidazole.

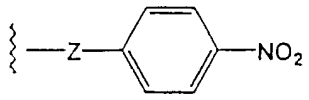
43. The method according to any one of claims 40 to 42, wherein said reaction is performed in a non-aqueous solvent comprising dimethylformamide.

44. A method for screening for a therapeutic agent, comprising:

(a) contacting a first target cell with a compound of any of claims 15 or 37 to 39, under conditions that favor the incorporation of the compound into the intracellular

54. A method for treating a pathology characterized by hyperproliferative cells in a subject comprising administering to the subject a compound of any of claims 15 or 37 to 39.

55. A method for screening for a therapeutic agent, comprising contacting a target cell with a compound of any of claims 15 or 37 to 39, wherein R^4 is:



which target cell favor the incorporation of the compound into the target cell, for the diagnostic purpose of detecting intracellular levels of thymidylate synthase.

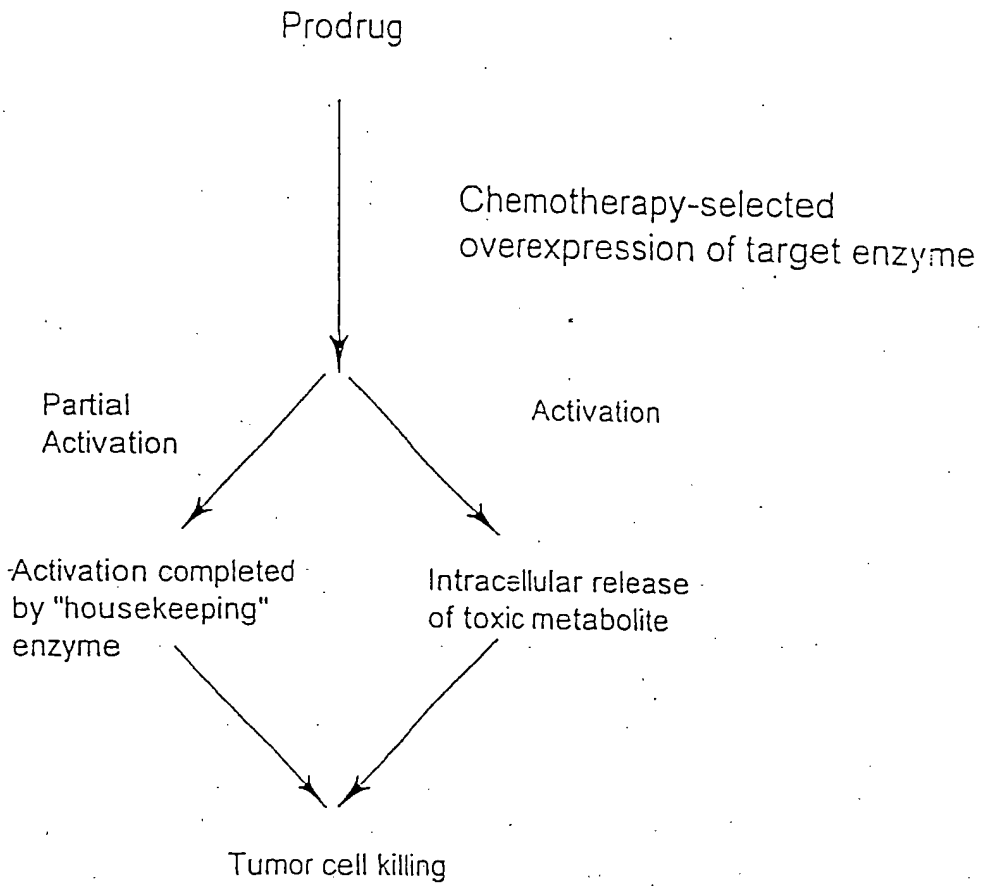

Prodrug
Chemotherapy-selected
overexpression of target enzyme
Partial
Activation
Activation
Activation completed
by "housekeeping"
enzyme
Intracellular release
of toxic metabolite
Tumor cell killing

Figure 2

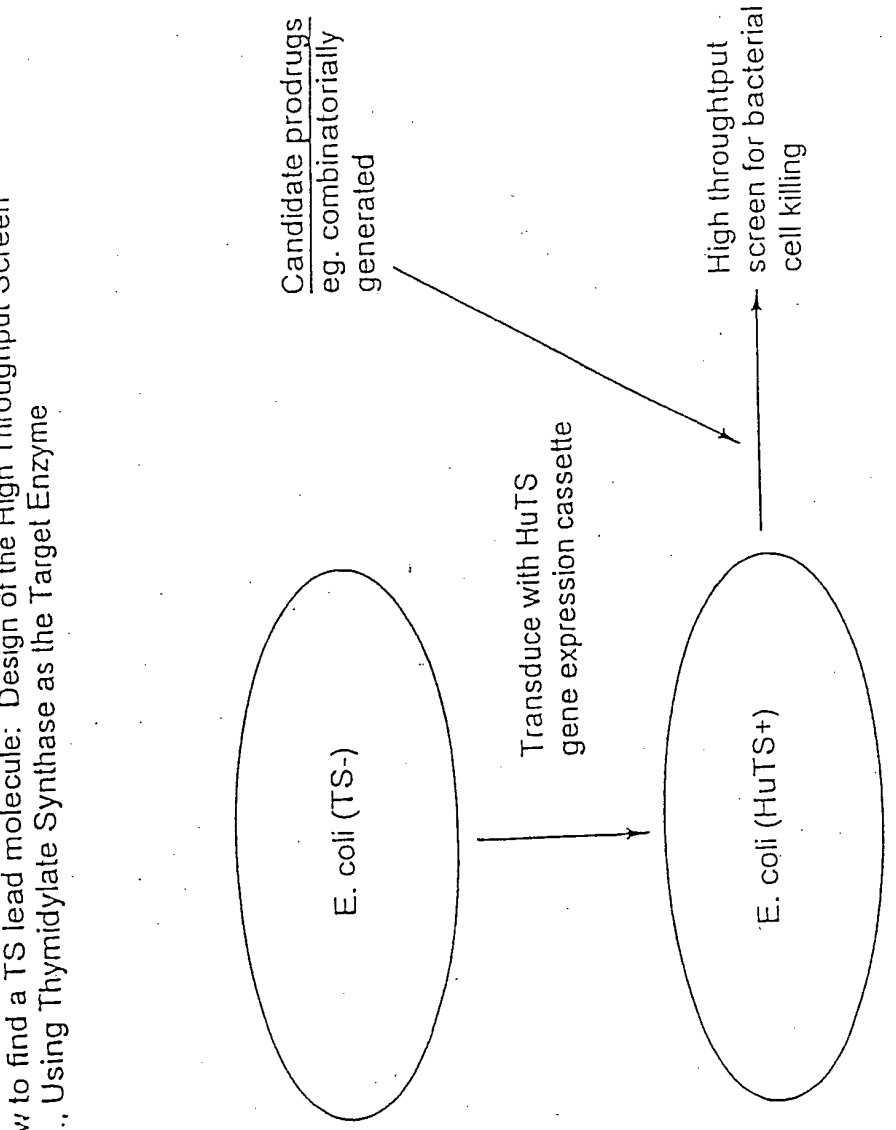

How to find a TS lead molecule: Design of the High Throughput Screen
e.g., Using Thymidylate Synthase as the Target Enzyme
Candidate prodrugs
eg. combinatorially
generated
High throughtput
screen for bacterial
cell killing
E. coli (TS-)
Transduce with HuTS
gene expression cassette
E. coli (HuTS+)

Figure 4

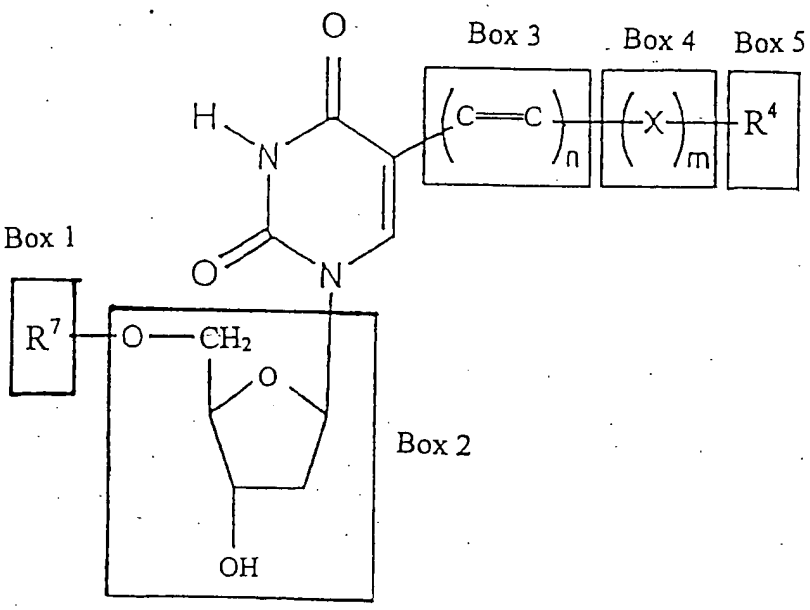


Figure 6

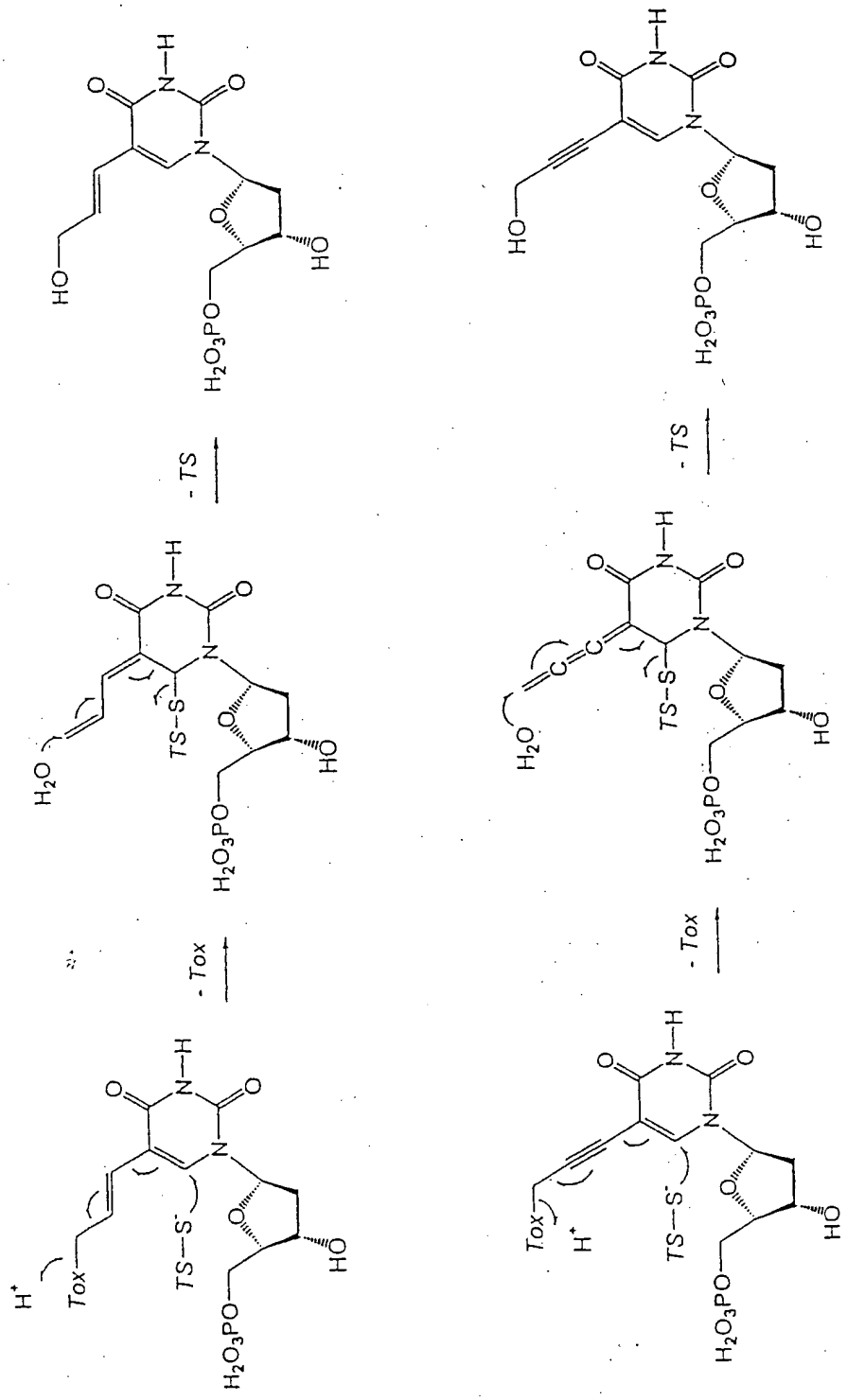


Figure 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/01332

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12N 9/10, 9/12, 5/18; C07H 19/04, 19/06, 19/044; A61K 51/00; A01N 43/04

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/193, 333; 424/1.73; 514/50, 51; 536/26.3, 28.53, 28.54, 28.55, 28.6